THE GENETIC ARCHITECTURE OF FEMALE REPRODUCTIVE MORPHOLOGY AND ITS INFLUENCE ON MALE AND FEMALE LIFE HISTORY IN YELLOW DUNG FLIES *SCATHOPHAGA STERCORARIA* (L.)

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. Nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich 2009

TO MY PARENTS

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Karin Thüler

CONTENTS

INTRODUCTION

Sexual selection drives evolutionary change to generate diverse morphological, behavioural and physiological adaptations (Parker, 1970; Birkhead & Møller, 1998; Birkhead, 2000). To understand such statements and the basic mechanisms this thesis is dealing with, I give here a brief review of the theoretical background of sexual selection and describe its main concepts.

Sexual selection can occur prior to, during and after copulation and appears in two forms: intrasexual selection involves characteristics which affect the outcome of competition among members of one sex for access to members of the other sex; intersexual selection favours the evolution of secondary sexual characteristics which improve the relative attractiveness of members of one sex to the other sex.

Pre-copulatory sexual selection includes all forms of sexual selection that occur before mating, including both active and passive female choice and competition between males (Le Roux, 2008). Intrasexual competition between males can lead to the evolution of conspicuous weaponry used during aggressive contests (e.g., antlers and horns in ungulates, Caro et al., 2003), or traits that assist in mate acquisition in the absence of direct contests (e.g., sensory apparatus in moths, Nakano et al., 2008). Intersexual competition favours traits that improve male attractiveness to females (zebra finch bill colour, Price & Burley, 1993; calling song in field crickets, Gray & Cade, 1999). Post-copulatory sexual selection involves sperm competition (intrasexual competition; Parker, 1970; reviewed in Simmons, 2001) and cryptic female choice, which is the ability of a female to influence the fertilization success of a mating partner (Eberhard, 1996) or the non-random storage or use of sperm as a result of female morphology, physiology or behaviour (Birkhead, 2000; Simmons, 2001). Although these divisions are somewhat arbitrary and individual traits may be involved in more than one process (reviewed in Kokko et al., 2003), the relative importance of these different forces in shaping diversity in sexually dimorphic traits remains a central question within evolutionary biology (Parker, 1970; Birkhead & Møller, 1998 and chapters within; Birkhead, 2000).

Sperm competition

If the sperm of more than one male compete directly for access to a female's eggs sperm competition occurs (Parker, 1970). Sperm traits that vary among males and may be relevant for sperm competition include ejaculate size (Simmons & Parker, 1992; Simmons, 2001) and sperm length (Miller & Pitnick, 2002, Simmons & Kotiaho, 2007). However, the benefits of long sperm seem to depend critically on female reproductive morphology. For example, male dung beetles (*Onthophagus taurus*) with short sperm have a fertilization advantage when females have large spermathecae (Simmons & Kotiaho, 2007). In contrast, in *Drosophila melanogaster* males from lines that had been selected for longer sperm had an advantage over males with short sperm, but only when mated to females with long seminal receptacles (Miller & Pitnick, 2002).

Female pre- and post copulatory mate choice

Female mate choice is supposed as one of the major processes of sexual selection (Darwin, 1871). Mate choice can be direct or indirect, active or passive (see below). In the field, we often observe competition between two (or more) males over a mating chance with a certain female (passive mate choice; e.g., in lekking species, like the little bustard *Otis tarda,* Black Grouse *Tetrao tetrix*, where males fight it out and females mate with the winner; Andersson, 1994). Such competition between males leads to intersexual selection for male traits which increase a male's ability to compete with other males for access to females (Holland & Rice, 1999). In contrast, through active pre-copulatory mate choice females are supposed to influence pre-copulatory male-male competition and may be able to choose with which male or males they want to mate (Hunt et al., 2005; active mate choice; e.g. in *Drosophila montana,* females prefer males with high-frequency sound pulses and actively choose between courting males that male with the preferred frequency; Suvanto, 1999). Nevertheless, it is quite obvious that female mate choice is largely influenced by the availability of mating partners (Jennions & Petrie, 1997).

An aspect of female mate choice that can be quantified is female preference (Boulcott et al., 2005). Darwin (1871) established the principle of female preferences for male ornaments (example of direct mate choice, where females select males based on variation in signalling traits, Genner, 2008). Further, Bakker & Pomiankowski (1995) showed that female mate preferences evolve adaptively and these are the major cause of variation in male sexual traits. The degree of female preference can be measured as the slope of the line relating female response to a male trait (e.g. orange area in male guppies; Houde & Endler, 1990; Jirotkul, 1999). If females show an extreme preference for a certain male trait co-variance between genes for the preference and genes for the preferred trait arises, because females with extreme mate preferences mate more frequently with males expressing extreme sexual traits (Fisher, 1930; Andersson & Simmons, 2006). After a female preference is established, choosing males with extensive sexual traits will produce sons carrying alleles for the trait and daughters carrying alleles for the preference (Maed & Arnold, 2004). Although, a female's preference is determined by the genes for this preference, there is individual variation in female preferences (Jennions & Petrie, 1997). Female mate preference can be influenced by several factors among and within populations (Verrell, 1999; Jennions & Petrie, 1997; Hunt et al., 2005); for example, by the individual behaviour of a certain female itself and the strength of the expressed preference for a specific male phenotype (Bakker et al., 1999), or it can be influenced by condition (Jennions & Petrie, 1997). Further, Hunt et al. (2005) suggested that resource acquisition may generate variation in mate choice behaviour.

Sexual selection can continue after copulation, a phenomenon referred to as post-copulatory sexual selection. Initially only males were thought to be able to influence post-copulatory sexual selection through competition between ejaculates (i.e., sperm; Parker, 1970). More recently, researchers have questioned the extent to which a female can influence the outcome of post-copulatory sexual selection. Thornhill (1983) first came up with the term "cryptic female choice", which was defined as any post-copulatory ability of a female to favour one male over another of the same species. Eberhard (1996) redefined the term as a post-copulatory sexual selection process through which females are able to

selectively favour the sperm of one male over sperm of other males (direct mate choice by choosing favoured sperm; indirect mate choice, where females generates sperm competition which allows cryptic female choice, because paternity is influenced by the male with high quality sperm; Gage, 2005) and kept the term "cryptic" because with the traditional methods it was not possible to detect a male's reproductive success through these processes and structures. .

Co-evolution of males and female traits

Female reproductive tract structures may coevolve with male reproductive traits through post-copulatory sexual selection (Parker, 1970; Birkhead & Møller, 1998 and chapters within; Birkhead, 2000; Arnqvist & Rowe, 2005; Snook, 2005), just as male external ornaments and weapons arise as a consequence of pre-copulatory selection imposed by females (Dixson & Anderson, 2001; House & Simmons, 2003; Presgraves et al., 1999; Miller & Pitnick, 2002; Minder et al., 2005; as explained above). Causes for phenotypic and genetic correlations can be distinguished at multiple levels which are not mutually exclusive and can influence traits simultaneously.

An important mechanism that may lead to genetic correlations (apart from female preferences for a male trait, see above) is intersexual conflict because of its influence on sexually dimorphic traits (reviewed in Arnqvist & Rowe, 2005). Intersexual conflict arises when males and females have different reproductive interests (females usually try to maximise paternal quality, while males usually try to maximise their share of paternity; Partridge et al., 1987*a*; Fowler & Partridge, 1989; Chapman et al., 2003; Pischedda & Chippindale, 2006). These different reproductive interests across the sexes may lead to sexually antagonistic selection if some alleles are favoured in females but not in males and *vice versa* (Arnqvist & Rowe, 2005).

There are two main forms of sexually antagonistic selection: intra- and interlocus sexual conflict (Parker & Partridge, 1998; see chapter 1 for examples). Intralocus sexual conflict is a conflict over which allele at a single locus is favored most in the two sexes, because alleles at one locus are expressed in both sexes but may be of different optima for male and female phenotypes (e.g. mating rate, where a high mating rate is harmful for females but of advantage for males; Rice & Chippindale, 2001; Arnqvist & Rowe, 2005). An intralocus conflict will then result (Halliday & Arnold, 1987), because selection in one sex will prevent adaptive evolution in the other sex (Rice, 1984; Lande, 1987; Parker & Partridge, 1998; Chippindale et al., 2001). This sexually antagonistic selection leads then to a negative intersexual genetic correlation for fitness-related traits.

In contrast, if alleles are expressed at different loci and there is a conflict over interactions between the sexes, an interlocus sexual conflict may occur (Arnqvist & Rowe, 2005). Interactions between locus A expressed in males (e.g. traits for grasping females for a longer copulation time), and another locus B expresses in females (traits to resist mating for no or a shorter copulation time), may favour alleles at only one locus, for example at locus A (male), because of the increased male ability to grasp; but, as a consequence, this will at the same time increase selection for female resistance to mating. The more males grasp the more females resist. These traits in question will therefore be selected in opposite directions and

this interlocus conflict may lead to a positive genetic correlation between the traits in question, if male and female traits are engaged in an antagonistic arms race, because each sex has adaptations promoting conflicting interests (Pischedda & Chippindale, 2006; Simmons & Siva-Jothy, 1998).

Different reproductive interests across the sexes may also occur for mate choice. Sexual selection predicts that both sexes should at least gain some benefits by exercising mate choice (Holland & Rice, 1999; direct benefits for females: e.g., territories, male parental care, reduced rate of sexually transmitted infections; indirect benefits for females: obtaining good genes from high-quality males; Andersson, 1994; Simmons, 2005; benefits for males: genetic benefits, Qvarnström, 2001; fitness and fecundity benefits, Preston et al. 2005); and both sexes may influence the outcome of paternity at any steps of their interaction (prior to and/or after copulation; Andersson, 1994). A good example of a mutual influence of males and females are the transferred seminal fluid with its sperm from males to females and the longevity of the sperm inside a female's tract. Although most males typically transfer large numbers of sperm during copulation, often only a tiny proportion reach the fertilization site (Chang, 1951; Hartman, 1957; Bedford, 1970; Austin, 1975; Smith & Yanagimachi, 1990; Suarez, 1987), because a female's reproductive tract is generally hostile to sperm (Birkhead et al., 1993) and they can actively or passively reduce sperm number in it (Davey, 1985; bruchid beetles: Eady, 1994; house flies: Degrugillier, 1985). Therefore, male sperm can be relatively short lived in a female's reproductive tract, and in response, males in some species produce and transfer substances to protect their ejaculates against female attacks (Chapman, 2001; Lung & Wolfner, 1999; Lung et al., 2002; Leopold et al., 1971; Merritt, 1989; Duvoisin et al., 1999). These substances often benefit only males and may harm females (e.g., Eberhard & Cordero, 1995; Bonduriansky et al., 2005). Alternatively we can hypothesize that differences in sperm viability in a female tract are not necessarily caused by spermicide of disfavoured sperm but rather by selective nourishing favoured sperm. Although the two functions are contrary, both indicate female reproductive influences on paternity.

However, exercising mate choice in general involves potential costs for females (e.g., searching for mating partners is time consuming, Jennions & Petrie, 1997; Schneider & Bürger, 2006; Ward et al., 2007; females experience higher predation risk as a result of mating activity, Pomiankowski, 1987; and lose energy; Kirkpatrick & Hall, 2004). Mate choice can also involve costly female choice structures. The complex reproductive tract of female yellow dung flies (which includes three or four sperm storage organs, known as spermathecae) may have evolved to provide females with more control over sperm storage and use, but building and maintaining complex reproductive traits probably represents a considerable cost for females (Ward, 2000; Ward et al., 2007; Jennions & Petrie, 1997; Schneider & Bürger, 2006; Pomiankowski, 1987; Kirkpatrick & Hall, 2004).

The balance of these potential costs and benefits mentioned above plays a large role in determining female investment in mate choice. It is therefore worth considering how this investment in costly choice structures may change with different levels of resource acquisition (Hunt et al., 2005). Both larval performance and maternal provisioning may exert a strong influence on resource acquisition (DeWitt & Scheiner, 2004). Resource acquisition is a primary driver of condition dependence (Kemp,

2008) and this is therefore an important factor to consider. Selection favours certain traits, or combinations of traits, over others (Birkhead & Møller, 1998; Simmons, 2001), for example, condition-dependent traits (Andersson & Simmons, 2006). Condition dependence is a form of developmental plasticity that links phenotypic trait expression to condition. There is a genetic basis of condition dependence (Bonduriansky & Rowe, 2005) and it is an important factor of resource investment in life-history traits. Further, variations found in female mate choice are condition dependent and related to the life-history traits (Hunt et al., 2005).

Research goal

The aim of this thesis was first to investigate the genetic architecture of female reproductive morphology (phenotypic and genetic correlations within and genetic correlations between the sexes) and the interactions important for its evolution and its influences on other male and female life history and morphological traits in the yellow dung fly *Scathophaga stercoraria* (L.). In other words, I studied genetic correlations between reproductive characters to see if there were hints about how reproductive morphology coevolves in response to changes in the opposite sex. I also aimed to give possible reasons for genetic correlations between several traits (e.g., common developmental origin or resource allocation pathway for some reproductive traits).

I was further interested in examine the costs of investing in complex female choice structures (spermathecae) that is thought to allow post-copulatory mate choice (e.g. sorting sperm from different males) and the consequences for post-copulatory sexual selection and implications for sexual conflict. I selected for a trait (from three to four spermathecae) that is arguably strongly involved in cryptic choice and analysed the effects of changing the female morphology on other traits in males and females.

To study the cost of investing in spermathecae in terms of its effect on other life history traits I separated the effect of the genetic background (selection lines) from the phenotypic expression of spermathecae and manipulated dam and offspring diet. I could then explore the relationship between resource acquisition, investment in post-copulatory mate choice, costly mate choice apparatus (spermathecae) and investment in other aspects of life-history in both males and females.

Finally I analysed a specific female trait (accessory gland fluid) in relation to its potential influences of female choice, control over sperm storage and use and therefore paternity.

STUDY ORGANISM

The yellow dung fly, *Scathophaga stercoraria,* is an important model system for studies of cryptic female choice and sperm competition (Parker et al., 1990; Ward & Simmons, 1991; Simmons & Parker, 1992; Ward, 1993, 2000). In the field, male yellow dung flies wait for females on or around fresh cowpats to seize them as soon as they arrive on the pat to oviposit; then copulation follows (Parker, 1970; 1978). After copulation, females lay their eggs in the fresh cow dung from which the larvae will feed. Adult flies emerge 2.5-5 weeks after pupation depending on temperature and food availability (Parker, 1970a). Although females seem unable to reject males and are therefore limited in their ability to choose a mating partner at this pre-copulatory stage (Ward, 2007), their complex reproductive tract apparently enables female to separate ejaculates from various males to a certain extent (Otronen et al., 1997) and to exert post-copulatory sexual selection on males that may relate to female reproductive morphology and behaviour (Ward, 2000).

The complex female reproductive tract of yellow dung flies usually includes three sperm storage organs (known as spermathecae); although in the field 2% of the females have four spermathecae. Each organ is associated with a duct that leads to the bursa copulatrix (Hosken, 1999). Females also possess paired accessory glands containing a fluid that may be involved in fertilization and egg laying (Leopold & Degrugillier, 1973; Davey, 1985). The fluid may also influence sperm viability (Hosken & Ward, 1999) by either harming sperm (i.e. acting as a spermicide; Hellriegel & Ward, 1998; Greef & Parker, 2000, Hosken et al., 2001; Bernasconi et al., 2002) or, alternatively, by nourishing sperm (Birkhead et al., 1993; Hellriegel & Ward, 1998; Hosken et al., 2001). Both of these functions may impose selection on males if the harm or nourishment applies selectively to a fraction of their mates.

OUTLINE OF THE CHAPTERS

Recent work, stimulated by the significant inter- and intra-specific variation found in reproductive traits, has suggested that both the morphology of sperm and the female reproductive morphology can influence paternity. Natural selection may favour female reproductive morphology that allows females to control mating, fertilization and paternity, and male reproductive traits may arise as counter-adaptations to subvert this control. Such co-evolution between female and male traits predicts the establishment of genetic correlations between male and female traits that interact during mating.

In **Chapter 1**, we aimed to clarify interactions important for the evolution of reproductive and morphological traits. We estimated the inheritance and phenotypic and genetic correlations between male and female reproductive tract characteristics in the yellow dung fly, *Scathophaga stercoraria*, using a modified nested half-sib breeding experiment including pedigree information. We expected positive phenotypic and genetic correlations for traits within sexes that are functionally integrated and/or linked via resource acquisition and in contrast, negative correlations between expensive life history traits that compete for metabolic resources.

Intersexual genetic correlations might be positive if structures share genes that have pleiotropic influences on development in both sexes (e.g. genes for reproductive effort expressed in both sexes), or if structures interact mechanistically (e.g., the length of sperm and the length of spermathecal ducts; Presgraves et al., 1999). In contrast, intersexual genetic correlations may be negative for fitness-related traits if the fitness rank of an allele is different across the sexes, e.g., because of differing optima for trait expression.

Post-copulatory sexual selection and mate choice are known to influence the evolution of male reproductive traits in many species. Female yellow dung flies seem to have some control over sperm storage and use due to their multiple spermathecae. Previous studies have demonstrated that females with four spermathecae may be better able to influence paternity than those with only three. Most females from the field have three spermathecae. In our study population we found around 2% of wild females have four.

In **Chapter 2** we established replicate lines and selected on the number of sperm storage organs (spermathecae). Selecting for spermatheca number is interesting because it represents a substantial jump in complexity that is naturally variable and has a presumably clear impact on the possibility to sort sperm. We document the results of our artificial selection experiment, as well as correlated response in male traits to this selection and examined genetic correlations between the expression of this reproductive apparatus and other phenotypic characters in males and females. Further, we investigate the potential underlying genetic basis of female choice according to spermatheca investment and investment in other aspects of morphology or life-history.

Adaptive female choice may account for some of the diversity in morphological and reproductive traits in males and females. But there is also variation in female mate choice that is due to condition-dependence,

which is an important factor affecting investment across life-history traits. Although females may benefit from mate choice, there are costs involved with exercising mate choice and building complex choice apparatus and limited resources may result in a trade-off in resource allocation. Females with large energy reserves may be better able to allocate resources to costly choice apparati and therefore to pay the costs of exercising cryptic choice than females in bad condition.

In **Chapter 3** we used the selection lines developed in the experiment described in Chapter 2 to explore how resource acquisition influenced the expression of the 4-spermatheca phenotype, and therefore the investment in costly post-copulatory mate choice apparatus (spermathecae). If the developmental history of a female (including provisioning by the mother) influences life-history and morphological traits, females reared on high quality diet should be able to allocate more resources to all life-history traits. These females may therefore invest more in choice (i.e., be more likely to express four rather than three spermathecae). Further, females with good genes for resource acquisition should be better able to produce a fourth spermatheca even if the resources are limited for mothers and/or offspring. By manipulating the larval diets of both dams and their offspring, we were able to examine the relationship between resource acquisition and allocation to spermathecae versus other aspects of life-history across artificial selection treatments and even across individuals within treatments that differed in the expression of the four-spermatheca phenotype.

Female yellow dung flies have a complex reproductive anatomy. This structure contains also paired accessory glands, filled with a fluid that is involved in fertilization and egg laying. Accessory gland secretions may also be help create a hostile insemination site and selectively kill sperm. Alternatively, female accessory gland fluid may actually keep sperm alive to avoid fertilization failure. Killing or conserving sperm are thus two possible but contrary functions of female accessory gland products. **Chapter 4** investigates these possible functions of accessory gland fluid. We exposed male yellow dung fly sperm to accessory gland fluid *in vitro* and investigated the fraction of sperm that were live or dead following this treatment.

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SUMMARY

Both intra- and intersexual selection can occur before and after mating. Pre-copulatory sexual selection includes any aspect of female choice and competition between males that occurs before mating. Post-copulatory sexual selection includes intrasexual selection in the form of sperm competition and intersexual selection in the form of cryptic female choice. These types of sexual selection may have important implications on the evolution of reproductive traits in both sexes, but/and individual traits may be involved in more than one process. Substantial inter- and intra-specific variation found in reproductive traits suggests that both male and female reproductive traits may be important in determining male reproductive success and structures in both sexes may coevolve. Such co-evolution predicts the establishment of genetic correlations between traits of the two sexes.

Intersexual conflict arises when females and males have different reproductive interests and may lead to sexually antagonistic selection. One potential source of conflict is the outcome of post-copulatory sexual selection; if, for example, a female favours one sire among her mates, her interests will oppose those of all the other males she has mated with. Overcoming the interests of these males may involve costly phenotypic structures. Because the balance of costs and benefits plays a large role in determining female investment in mate choice, it is worth considering how this investment may change with different levels of resource acquisition and maternal effects.

Chapter 1

Inter- and intra-specific variation found in reproductive traits has suggested that the morphology of sperm and the female reproductive tract can both influence paternity, but the evolutionary implications of this variation remain unclear. Natural selection may favour female reproductive morphology that allows females to control mating, fertilization and paternity; and diverse male reproductive traits may arise as counter-adaptations to subvert this control. Such co-evolution between female and male traits predicts the establishment of genetic correlations between male and female traits that interact during mating.

To examine the consequences for genetic architecture of the reproductive interactions between male and female yellow dung flies, we analysed the heritabilities of and phenotypic and genetic correlations between male and female reproductive tract characteristics in the yellow dung fly, *Scathophaga stercoraria*, using a modified nested half-sib breeding experiment including pedigree information. For traits within sexes that are functionally integrated and/or linked via resource acquisition we expected positive phenotypic and genetic correlations. In contrast we expected negative correlations between expensive life history traits that compete for metabolic resources.

We found significant heritabilities for most reproductive traits investigated in both females and males and significant positive phenotypic and genetic correlations between the size of the spermathecae and the length of spermathecal ducts, as well as between the size of the accessory glands and their ducts, suggesting a common developmental origin or resource allocation pathway for some reproductive traits. We also found strong negative intersexual genetic correlations between male body size and the size of

spermathecae, male body size and accessory glands, and between female spermatheca size and male testis size. This last negative genetic correlation between female spermatheca size and male testis size is consistent with the presence of intersexual ontogenetic conflict between these structures, perhaps involving sexual conflict over the control of sperm storage and use. We discuss alternative explanations for this genetic co-variance and directions for future research that might distinguish between these alternatives.

Chapter 2

Pre- and post-copulatory sexual selection and mate choice may influence the evolution of reproductive traits in many species, but the nature of selection on female traits that influence post-copulatory selection is much less well understood. Female yellow dung flies have remarkable control over sperm storage and use, presumably caused in part by of their multiple spermathecae. Most females from the field have three spermathecae; in our study population we found around 2% of wild females have four. Previous studies have demonstrated that females with four spermathecae may be better able to influence paternity than those with only three.

We established replicate lines and selected on spermathecae number. An increase in spermatheca number is a considerable jump in complexity that is naturally variable and may have an impact on the efficiency of sperm sorting. This represents a unique opportunity to study how genetic variation for female traits associates with genetic variation for male traits.

We successfully increased the fraction of females with four spermathecae to about 50% after eight generations of selection and examined the correlated response in male traits to this selection and the genetic correlations between the expression of the reproductive apparatus and other phenotypic characters in males and females. We found a trade-off between spermatheca size/volume and number, although the overall size/volume of females with four spermathecae is effectively larger (representing a larger overall storage space) than females with three spermathecae. We also found evidence for other trade-offs between investment in this trait and investment in other aspects of morphology or life-history. Further, we found that females in 4-spermatheca-lines laid significantly larger clutches than females in 3-spermatheca-lines. Clutch size decreased with increase of spermathecae size/volume, possibly representing a cost in form of a trade-off in resource allocation. The relationship between investment in an important choice structure (spermathecae number) and other morphological traits in females and males may have consequences for post-copulatory sexual selection and implications for sexual conflict.

Chapter 3

Adaptive female choice may account for some of the diversity in morphological and reproductive traits in males and females. Variation in female mate choice due to condition-dependence is an important factor of resource investment in life-history traits. Although females may benefit from mate choice, there are costs involved with exercising mate choice and building complex choice apparatus and limited resources may result in a trade-off in resource allocation. Females with large energy reserves may be better able to

allocate resources to costly choice apparatus and therefore to pay the costs of exercising cryptic choice than females in bad condition. The ability to sort sperm could therefore also be condition (i.e. nutrition) dependent.

We used the selection lines established in chapter 2 to explore how resource acquisition influenced the expression of the 4-spermatheca phenotype, and to examine the effects of the developmental history of a female (including provisioning by the mother) on the investment in costly choice apparatus and therefore possibly the ability to exercise choice. We manipulated the larval diet for both dams and their offspring and examined the relationship between resource acquisition, investment in costly mate choice apparatus (spermathecae) and investment in other aspects of life-history in both males and females.

The expression of four spermathecae (in 4-spermathecae-lines) had a significant negative effect on female fecundity. Further, this expression is condition-dependent, because a high quality dam diet influenced the expression of a fourth spermathecae positively (while offspring diet had no effect). Maternal condition seemed to have a stronger effect on spermatheca number than offspring condition itself. However, neither dam nor offspring diet seemed to have any significant effect on male traits. Although there was considerable genetic variation in spermathecae number, the ability to sort sperm may be related to nutrient acquisition. Fluctuations in the ability to acquire resources may be an important factor maintaining genetic variation in costly female choice (apparatus) and thus potentially driving heterogeneity in sexual selection on male sexual traits. A trade-off in investment between choice apparatus and fecundity or other life history characters may reveal that such allocation decisions are a strong constraint on the evolution of choice.

Chapter 4

Female and male reproductive traits are thought to coevolve through pre- and post-copulatory sexual selection and sexual conflict. Although males typically transfer large numbers of sperm during copulation, only a tiny proportion reach the fertilization site because females often actively or passively reduce sperm number in their reproductive tract. Female yellow dung flies (*Scathophaga stercoraria)* have paired accessory glands that are filled with a fluid involved in fertilization and egg laying. Accessory gland secretions may be used to create a hostile insemination site and selectively kill sperm. Alternatively, female accessory gland fluid may nourish sperm to avoid fertilization failure. Killing or conserving sperm are thus two possible but contrary functions of female accessory gland products. We investigated the function of accessory gland fluid when interacting with male sperm *in vitro* and investigated the remaining live or dead sperm. Significantly more sperm remained alive when exposed to accessory gland fluid than when exposed to buffer only (66% vs. 47%).

We conclude that female accessory gland fluid serves primarily to conserve rather than kill male sperm. If this can be done selectively, our results are consistent with females playing an important part in influencing paternity, but further research is needed to identify the various components of this fluid and the mechanisms underlying variation in sperm viability and any possible selective influence on it that females might have.

ZUSAMMENFASSUNG

Intra- und intersexuelle Selektion kann sowohl vor als auch nach der Paarung, bzw. Kopulation, auftreten. Die sexuelle Selektion, welche vor der Kopulation auftritt, beinhaltet alle Aspekte der weiblichen Partnerwahl, sowie das Wettbewerbsverhalten der Männchen, welches der Kopulation vorangeht. Sie kann unterteilt werden in intrasexuelle Selektion in Form von Spermien-Konkurrenz und intersexuelle Selektion in Form von kryptischer Weibchen-Wahl (Partnerwahl). Diese beiden Typen der sexuellen Selektion haben wichtige Auswirkungen auf die Evolution der Geschlechtsmerkmale in Weibchen und Männchen, jedoch können die einzelnen Merkmale eines Individuums in mehr als einen Prozess involviert sein.

Eine beachtliche Fülle an inter- und intraspezifischer Variation in diesen Merkmalen deutet darauf hin, dass sowohl männliche und wie auch weibliche Geschlechtsmerkmale eine wichtige Rolle in der Bestimmung des männlichen Fortpflanzungserfolges spielen. Strukturen in beiden Geschlechtern können demnach ko-evoluieren. Solche Ko-Evolutionen prognostizieren das Entstehen von genetischen Korrelationen zwischen Merkmalen beider Geschlechter.

Intersexueller Konflikt entsteht wenn Weibchen und Männchen unterschiedliche Fortpflanzungsinteressen haben, was zu antagonistischer sexueller Selektion führen kann. Eine mögliche Konfliktquelle ist das Ergebnis der sexuellen Selektion nach der Paarung wenn beispielsweise ein Weibchen ein Männchen gegenüber anderen Partnern bevorzugt. Ihre eigenen Interessen werden so denen aller anderen Männchen mit denen sie sich paarte, gegenüberstehen. Um diesen Konflikt zu bewältigen, werden aufwendige phänotypische Strukturen ausgebildet. Die Balance zwischen Kosten und Gewinn bei einer Kopulation spielt eine wichtige Rolle in der Bestimmung, welche Investitionen ein Weibchen in die Partnerwahl steckt. Es ist deshalb wertvoll, mögliche Veränderungen dieser Investitionen in Bezug auf eine Ressourcen-Veränderung zu betrachten und die Einflüsse, welche auch die Mutter ausüben könnte, einzubeziehen.

Kapitel 1

Die inter- und intraspezifische Variation in den Geschlechtsmerkmalen besagt, dass sowohl die Spermien-Morphologie als auch der weibliche Geschlechtsapparat eine mögliche Vaterschaft beeinflussen kann. Die evolutiven Implikationen dieser Variationen bleiben jedoch unklar. Wenn die natürliche Selektion die weibliche Geschlechtsmorphologie bevorzugt, wird den Weibchen eine mögliche Kontrolle über die Partnerwahl, die Befruchtung und die Vaterschaft ermöglicht. Verschiedene männliche Geschlechtsmerkmale können deshalb als Gegen-Adaptationen entstehen, um diese Kontrolle zu untergraben. Solche Ko-Evolutionen zwischen männlichen und weiblichen Merkmalen, welche während der Paarung interagieren, prognostizieren die Entstehung genetischer Korrelationen zwischen den betreffenden Merkmalen.

Um diese Konsequenzen für die genetische Architektur solcher geschlechtlichen Interaktionen zwischen Männchen und Weibchen zu untersuchen, haben wir die Vererblichkeit (Heritabilität) von und die phänotypischen und genetischen Korrelationen zwischen männlichen und weiblichen Charakteristiken

des Geschlechtsapparates der gelben Dungfliege, *Scathophaga stercoraria*, analysiert. Wir benutzten ein Voll- und Halbgeschwister Experiment, welches die Informationen des Stammbaums beinhaltet. Für Merkmale innerhalb der Geschlechter, welche funktional integriert sind oder verbunden via Ressourcen-Aufnahme, haben wir positive phänotypische und genetische Korrelationen erwartet. Im Gegensatz dazu erwarteten wir eine negative Korrelation zwischen aufwändigen fortpflanzungsrelevanten Merkmalen, welche um metabolische Ressourcen konkurrenzieren.

Wir fanden eine signifikante Heritabilität für fast alle Geschlechtsmerkmale beider Geschlechter. Weiter fanden wir signifikante positive phänotypische und genetische Korrelationen zwischen der Grösse der Spermatheken und der Länge der Spermatheken-Dukten, sowie zwischen der Grösse der Anhangdrüsen und deren Dukten, was einerseits auf einen gemeinsamen Entwicklungsursprung hindeuten könnte oder anderseits darauf, dass diese Merkmale in gleicherweise mit Ressourcen versorgt werden.

Weiter fanden wir eine starke negative intersexuelle genetisch Korrelation zwischen der Körpergrösse der Männchen und der Grösse der weiblichen Spermatheken, der Körpergrösse der Männchen und der Grösse der weiblichen Anhangdrüsen und zwischen der Grösse der weiblichen Spermatheken und den (männlichen) Hoden. Diese letzte negative genetische Korrelation deckt sich mit dem Vorhandensein eines intersexuellen ontogenetischen Konflikts zwischen diesen Strukturen. Wahrscheinlich weil ein sexueller Konflikt über die Kontrolle der Spermienspeicherung und deren Verwendung involviert ist. Für diese genetischen Ko-Varianzen diskutierten wir ebenfalls alternative Erklärungen, sowie Richtungen und Möglichkeiten für zukünftige Forschung, wie diese Alternativen unterschieden werden könnten.

Kapitel 2

In vielen Arten beeinflusst die pre- und postkopulatorische sexuelle Selektion sowie die Partnerwahl die Evolution der Geschlechtsmerkmale. Die Wirkung der Selektion auf weibliche Merkmale ist jedoch kaum bekannt. Weibliche Gelbe Dungfliegen haben eine beachtliche Kontrolle über die Spermienspeicherung und deren Verwendung, vermutlich durch ihre multiplen Spermatheken. Die meisten Weibchen im Feld haben drei Spermatheken ausgebildet. In unserer Studie fanden wir etwa 2% der Weibchen mit vier Spermatheken. Frühere Studien demonstrierten, dass Weibchen mit vier Spermatheken eine bessere Fähigkeit besitzen, um die Vaterschaft zu beeinflussen, als solche mit nur drei.

Wir etablierten Selektionslinien (mit je drei Replikate) und selektierten auf die Spermatheken-Zahl. Eine erhöhte Spermatheken-Zahl ist ein beachtlicher Sprung in der Komplexität, welche natürlich variabel ist und einen Einfluss auf die Effizienz der Spermien-Auswahl haben könnte. Weiter repräsentiert dies eine einmalige Gelegenheit herauszufinden, wie genetische Variation der weiblichen Merkmale mit der genetischen Variation der männlichen Merkmale in Verbindung steht.

Wir konnten den Anteil an Weibchen mit vier Spermatheken nach acht Generationen erfolgreich auf ca. 50% erhöhen. Weiter haben wir die korrelierte Antwort der männlichen Merkmale auf diese Selektion analysiert und die genetischen Korrelationen zwischen der Ausprägung des Geschlechtstrakts und anderen phänotypischen Charakteristiken in Männchen und Weibchen untersucht.

Wir fanden einen Konflikt (trade-off) zwischen der Grösse und dem Volumen der Spermatheken und deren Anzahl, obwohl die Gesamtgrösse und das Gesamtvolumen der Weibchen mit vier Spermatheken effektiv grösser ist (grösserer Speicherplatz) als bei den Weibchen mit nur drei Spermatheken. Weiter fanden wir Anzeichen für andere Konflikte (trade-offs) zwischen der Investition in eine erhöhte Anzahl Spermatheken und anderen Aspekten der Morphologie und fortpflanzungsrelevanten Merkmalen. Interessanterweise haben Weibchen in den 4-Spermatheken-Selektionslinien signifikant mehr Eier gelegt (grössere Gelege) als Weibchen in den 3-Spermatheken-Selektionslinien. Die Gelegegrösse nahm ab mit zunehmender Spermathekengrösse, bzw. zuhnehmendem Spermathekenvolumen. Dies repräsentiert einen möglichen Aufwand (Kosten) in Form eines Konflikt in der Ressourcenbeschaffung und/oder -verteilung. Dieser Zusammenhang zwischen der Investition in wichtige Strukturen der Partnerwahl (Spermatheken-Zahl) und anderen morphologischen Merkmalen in Weibchen und Männchen kann Konsequenzen für post-kopulatorische sexuelle Selektion und Auswirkungen für den Sexuellen Konflikt haben.

Kapitel 3

Weibliche Partnerwahl ist anpassungsfähig und könnte für die Vielfalt in morphologischen und geschlechtlichen Merkmalen in Männchen und Weibchen beitragen. Die Variation in weiblicher Partnerwahl aufgrund von konditionsbedingten Einflüssen (Umwelteinflüsse) ist ein wichtiger Faktor für die Investition der Ressourcen in fortpflanzungsrelevante Merkmale. Obwohl Weibchen von der Partnerwahl profitieren können, sind Kosten damit verbunden. Der Aufbau solch komplexer Partnerwahl-Strukturen und die limitierten Ressourcen können in einem Konflikt in der Ressourcen-Verteilung enden. Weibchen mit grossen Energie-Reserven scheinen besser fähig zu sein, Ressourcen in aufwändige und kostspielige Strukturen zu investieren und deshalb diese Kosten der Partnerwahl zu bezahlen, bzw. auszugleichen, als Weibchen in schlechten Konditionen. Die Fähigkeit Spermien zu sortieren könnte deshalb Konditionsbedingt sein (z. B. Nahrungsbedingt).

Wir benutzen hierfür die gleichen Selektionslinien, welche wir in Kapitel 2 etablierten, um herauszufinden, wie die Ressourcen-Beschaffung die Ausbildung des 4-Spermatheken-Phänotyps beeinflussen kann. Weiter prüften wir die möglichen Effekte der weiblichen Entwicklungsgeschichte (einschliesslich der Versorgung durch die Mutter) auf das Investieren in aufwendige Auswahl-Strukturen und somit auf die Fähigkeit zwischen möglichen Partnern zu wählen. Wir manipulierten die larvale Diät (Nahrung) der Mütter sowie der Nachkommen und untersuchten das Verhältnis zwischen Ressourcen-Beschaffung, Investierung in aufwändige Partnerwahl-Strukturen (Spermatheken) und die Investierung in andere Aspekte fortpflanzungsrelevante Merkmale in Männchen und Weibchen.

Die Ausbildung von vier Spermatheken (in 4-Spermatheken-Linien) hatte einen signifikanten negativen Effekt auf die Fruchtbarkeit der Weibchen. Weiter ist diese Ausbildung abhängig von den Ressourcen, weil eine qualitativ hochstehende Diät der Mütter die Ausbildung einer vierten Spermatheke positive beeinflusste (während die Diät der Nachkommen keinen Einfluss zeigte). Die mütterlichen Konditionen scheinen einen stärkeren Effekt auf die Spermatheken-Zahl zu haben als die Konditionen der

Nachkommen selber. Doch weder die mütterliche, noch die Diät der Nachkommen schien einen signifikanten Effekt auf die männlichen Merkmale auszuüben.

Obwohl es eine beachtliche genetische Variation in der Spermatheken-Zahl gibt, die Fähigkeit Spermien zu sortieren könnte mit der Nahrungsbeschaffung und deren Aufnahme zusammenhängen. Schwankungen in der Fähigkeit Ressourcen zu beschaffen, könnte relevant für die Aufrechterhaltung dieser genetischen Variation in aufwändigen weiblichen Auswahl-Strukturen sein und deshalb möglicherweise eine Heterogenität in der sexuellen Selektion auf die männlichen Merkmale ausüben. Ein Konflikt (trade-off) in der Investition zwischen Partnerwahlstrukturen und Fruchtbarkeit oder anderen Charakteristiken der fortpflanzungsrelevanten Merkmale, könnte offenlegen, dass solche Beschaffungs-Entscheide eine starke Bedingung auf die Evolution der Partnerwahl darlegen.

Kapitel 4

Weibliche und männliche Geschlechtsmerkmale ko-evoluieren durch pre- und post-kopulatorische sexuelle Selektion und sexuellen Konflikt. Obwohl Männchen während der Kopulation typischerweise eine grosse Anzahl an Spermien transferieren, erreicht jedoch nur ein Bruchteil davon den Befruchtungsort, weil die Weibchen oft aktiv oder passiv die Spermienzahl in ihrem Geschlechtstrakt reduzieren. Die Weibchen der Gelben Dungfliege weisen gepaarte Anhangdrüsen auf, welche mit einer Flüssigkeit gefüllt sind, welche in die Befruchtung und das Eierlegen involviert ist. Die Sekretion dieser Anhangdrüsen können dazu benutzt werden, einen unfreundlichen Befruchtungsort zu kreieren und selektiv Spermien abzutöten. Andernfalls kann diese Flüssigkeit auch für die Ernährung der Spermien benutzt werden, um einen Befruchtungsmisserfolg zu vermeiden. Das Abtöten oder Konservieren (Ernähren) der Spermien sind zwei mögliche jedoch gegenteilige Funktionen dieser weiblichen Anhangdrüsenflüssigkeit.

Wir untersuchten die Funktion der Anhangdrüsenflüssigkeit beim interagieren mit männlichen Spermien *in vitro* und untersuchten weiter die übrigbleibenden lebenden oder toten Spermien. Signifikant mehr Spermien blieben am Leben wenn sie der Anhangdrüsenflüssigkeit ausgesetzt waren als wenn sie nur mit Puffer versetzt wurden (66% vs. 47%).

Wir folgerten daraus, dass die weibliche Anhangdrüsenflüssigkeit in erster Linie dazu dient, die Spermien zu konservieren und nicht abzutöten. Wenn dies von den Weibchen selektiv getan werden kann, unterstützt unser Resultat die Annahme, dass die Weibchen eine wichtige Rolle in der Beeinflussung der Vaterschaft spielen. Weitere Forschung ist jedoch nötig, um die verschiedenen Komponenten dieser Flüssigkeit zu identifizieren und die Mechanismen zu untersuchen, welche der Variation der Lebensfähigkeit der Spermien unterliegen und welche selektiven Einflüsse die Weibchen darauf haben könnten.

GENETIC AND ENVIRONMENTAL SOURCES OF CO-VARIANCE AMONG INTERNAL REPRODUCTIVE TRAITS IN YELLOW DUNG FLIES

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ABSTRACT

Substantial inter- and intra-specific variation is found in reproductive traits, but the evolutionary implications of this variation remain unclear. One hypothesis is that natural selection favours female reproductive morphology that allows females to control mating and fertilization, and that diverse male reproductive traits arise as counter-adaptations to subvert this control. Such co-evolution between female and male traits predicts the establishment of genetic correlations between male and female traits that interact during mating. To clarify the interactions important for the evolution of reproductive morphology, we measured phenotypic and genetic correlations between male and female reproductive tract characteristics in the yellow dung fly, *Scathophaga stercoraria*, using a modified nested half-sib breeding experiment including pedigree information. We found significant heritabilities for the size of most reproductive tract traits investigated in both females (sperm storage organs, spermathecal ducts, accessory glands and accessory gland ducts) and males (testis size but not sperm length). Significant positive phenotypic (and genetic) correlations between spermatheca size and spermathecal duct length, as well as between accessory gland size and accessory gland duct length, suggest a common developmental origin or resource allocation pathway for some reproductive traits. Interestingly, we found strong negative intersexual genetic correlations between male body size and (female) spermatheca and accessory gland size, as well as between female spermatheca size and male testis size. This last genetic correlation is intriguing because it indicates the potential for these reproductive traits to coevolve. It is also consistent with potential intersexual ontogenetic conflict between these structures mediated by sexual conflict over the control of sperm storage and use. We discuss alternative explanations for this genetic co-variance and directions for future research that might distinguish between these alternatives.

INTRODUCTION

Biologists have been studying how sexually dimorphic traits have evolved through sexual selection for a long time, but the evolutionary mechanisms involved remain the subject of debate (Birkhead & Møller, 1998; Birkhead, 2000; Arnqvist & Rowe, 2005; Snook, 2005). Sexual selection encompasses a range of evolutionary processes, some of which take place before mating (e.g., competition between males), while others take place afterwards (i.e., sperm competition; Parker, 1970; cryptic female choice, Eberhard, 1996). Individual traits may be involved in more than one process (reviewed in Kokko et al., 2003) and the relative importance of these different forces in shaping diversity in sexually dimorphic traits remains a central question within evolutionary biology (Parker, 1970; Birkhead & Møller, 1998; Birkhead, 2000).

Sperm competition occurs when the sperm of more than one male compete directly for access to a female's eggs (Parker, 1970). Although substantial variation in sperm morphology across males has long been recognized, evolutionary biologists have only recently started to investigate the implications of this and other variation in male reproductive characteristics (Snook, 2005). Aspects of morphology that vary among males and may be relevant for sperm competition include ejaculate size (Simmons & Parker, 1992; Simmons, 2001) and sperm length (Miller & Pitnick, 2002, Simmons & Kotiaho, 2007). However, the benefits of long sperm seem to depend critically on female reproductive morphology. In *Drosophila melanogaster*, for example, males from lines that had been selected for longer sperm had an advantage over males with short sperm, but only when mated to females with long seminal receptacles (Miller & Pitnick, 2002). Similarly, male dung beetles (*Onthophagus taurus*) with short sperm have a fertilization advantage, but only within females with large spermathecae (Simmons & Kotiaho, 2007).

The female influence over sperm competition illustrated by variation in reproductive morphology may represent adaptive mate choice. After multiple inseminations, females may favour the ejaculates of a subset of males (a phenomenon known as cryptic female choice, which is defined as the non-random storage or use of sperm as a result of female morphology, physiology or behaviour; Birkhead, 2000; Simmons, 2001), and the outcome of this choice may depend on male reproductive characters. There is now increasing evidence that male reproductive anatomy (through its role in producing, storing and using sperm) can evolve through sexual selection just like ornaments and weapons (Dixson & Anderson, 2001; House & Simmons, 2003; Presgraves et al., 1999; Miller & Pitnick, 2002; Minder et al., 2005). But while pre-copulatory mate-choice is now relatively well understood, we still know remarkably little about the evolutionary consequences of post-copulatory sexual selection. This is at least partly because of practical difficulties in studying interactions that occur within the female reproductive tract, but also because the adaptations involved are typically less extravagant than the conspicuous external traits involved in pre-copulatory interactions.

In addition to the above-mentioned classical mechanisms of pre- and post-copulatory sexual selection (sperm competition, cryptic female choice), the last decade has seen substantial interest in theory and empirical work supporting the role of intersexual conflict in driving sexual dimorphisms (reviewed in Arnqvist & Rowe, 2005). Intersexual conflict arises when the fitness benefits of a mating pair diverge

(Chapman et al., 2003; Pischedda & Chippindale, 2006). Whereas typically females aim at maximising paternal quality, it is in the male's interest to maximize his share of paternity, whatever the cost to female lifetime reproductive success. For example, in *Drosophila melanogaster* male seminal fluid up-regulates a female's egg-laying rate and reduces her desire to remate, but also causes a decrease in her lifespan and ultimately her lifetime reproductive success (Partridge et al., 1987*a*; Fowler & Partridge, 1989; Chapman et al., 1993; Singh et al., 2002; Pitnick & Garcia-Gonzalez, 2002; Chapman & Davies, 2004). Different reproductive interests across the sexes may therefore lead to sexually antagonistic selection if some alleles are favoured in females but not in males and *vice versa* (Arnqvist & Rowe, 2005).

We can distinguish two forms of sexually antagonistic selection: intralocus and interlocus conflict (Parker & Partridge, 1998). Interlocus sexual conflict may arise if there is a conflict over interactions between males and females mediated by alleles expressed at separate loci (Arnqvist & Rowe, 2005). As an example, we can consider a hypothetical scenario in which conflict over mating frequency in water striders is mediated by alleles at two loci (Arnqvist & Rowe, 2002; Han & Jablonski, 2009). Imagine that the outcome of any attempted copulation is determined by an interaction between a locus A, which is expressed in males (e.g., traits for grasping females), and another locus B expressed in females (e.g., which defines female resistance to mating). Alleles may be favoured at locus A, for example, because they increase the male ability to grasp, and therefore spread in the population, but this will consequently increase selection for female resistance to mating. To the extent that there is an evolutionary response to this selection it will in turn create selection on locus A (and any other loci in males that affect the ability to grasp females). This intersexual interlocus conflict may continue and a replacement of alleles at one or many interacting loci in both females and males will arise (Rice & Holland, 1997). Such interlocus conflict may lead to a genetic correlations between the traits in question, if male and female structures are engaged in an antagonistic arms race and each sex has adaptations promoting conflicting interests (positive genetic correlation: e.g., if male traits for grasping females and female resistance to mating or if long sperm circumvent female control over fertilization and long ducts allow females to restore it; negative genetic correlation: if male and female traits are under opposing selection because of their different reproductive interests; Pischedda & Chippindale, 2006; Simmons & Siva-Jothy, 1998; reviewed in Gillott, 2003; e.g., sexual dimorphism in human hip width as a fitness function, which is controlled polygenically and many of the contributing loci were expressed in females and males, but through disruptive selection in past times on hip size, wider hips were favoured in females but not in males; Rice & Chippindale, 2001).

Intralocus sexual conflict occurs when selection favours different alleles at a single locus for males versus females (e.g. tail length in songbirds, mating rate). Each allele therefore moves one sex towards and the other sex away from its phenotypic optimum (Rice & Chippindale, 2001; Arnqvist & Rowe, 2005). For example, if the mating rate in both sexes is affected by a single locus and there is selection for higher mating rate in males and lower mating rate in females, then intralocus conflict will result (Halliday & Arnold, 1987). Selection in one sex will then impede adaptive evolution in the other sex (Rice, 1984; Lande, 1987; Parker & Partridge, 1998; Chippindale et al., 2001) and a negative intersexual

genetic correlation for fitness may occur, because the same allele has high fitness in males but low fitness in females. In other words, the differences in how both sexes maximize their evolutionary fitness can lead to intra-locus sexual conflict in which genes delivering fitness benefits to one sex are costly when expressed in the other (Zajitschek et al., 2007). Therefore, in this case, fitness plays a central role in patterns of covariances. In principle, a negative correlation could also arise from conventional sexual selection, but in this case this would occur only if one of the traits covaried negatively with fitness.

The fact that there can be different optima for traits shared by males and females may help explain the pervasiveness of sexual dimorphism, and the co-variance between reproductive traits may help identify the evolutionary mechanism responsible for sexual dimorphism (positive genetic correlations may be due to interlocus sexual conflict; negative genetic correlation between fitness-related traits may be due to intralocus sexual conflict). However, disentangling the evolutionary forces that have led to any particular sexually dimorphic trait is challenging, because the various forces that promote divergence between the sexes can act simultaneously and often produce similar phenotypic effects (Vincent & Herrel, 2007) and patterns of genetic co-variance (Steven et al., 2007). In many cases more than one of these forces will act on different parts of a single system. More proximate explanations can also be invoked to explain patterns of trait co-variance within and across the sexes. For example, when two traits are functionally integrated (e.g., genitalic apparati that need to fit together during copulation), they will necessarily covary in size in order to effectively function (Cheverud, 1996). Similarly, if two structures arise from a shared cluster of precursor cells, they will covary in size because increased investment in these precursors will result in positive correlations, or because local trade-offs in metabolic resources during development result in negative correlations. Good examples for intrasexual functional integration are ejaculates in which there are multiple components that have to cooperate to fulfil several concurring or sometimes competing functions (Moore et al., 2004). An example for intersexual developmental integration are genes for reproductive effort expressed in both sexes (Zajitschek et al., 2007).

In the current study we will look for correlations as possible signs of co-evolution between reproductive characters. We investigated a series of reproductive traits in the yellow dung fly *Scathophaga stercoraria,* a model system for studies of cryptic female choice and sperm competition (Parker et al., 1990; Ward & Simmons, 1991; Simmons & Parker, 1992; Ward, 1993, 2000). Male yellow dung flies wait for females on or around cow dung pats, seize and then copulate with them as they arrive at the pat to lay their eggs (Parker, 1970; 1978). Females seem unable to reject males, and although they are thus limited in their ability to choose with which male to mate, they may nevertheless be able to exert post-copulatory sexual selection. Indeed, there is evidence for subtle, cryptic female effects on fertilization success relating to female reproductive morphology and behaviour (Ward, 2000).

The female reproductive tract of yellow dung flies consists of paired accessory glands containing a fluid that may influence sperm viability (Hosken & Ward, 1999), and three or in rare cases four sperm storage organs (spermathecae), each associated with a duct that leads to the bursa copulatrix (Figure 1). During copulation, a male's aedeagus is inserted into the muscular bursa copulatrix near the spermathecal duct openings, where sperm are released. Although it is not yet clear if sperm move themselves or to what

extent females can propel or hinder this movement, the female appears to be actively involved in transporting sperm from the bursa and up the spermathecal ducts to the spermathecae, where they are stored until fertilisation occurs during oviposition (Ward, 1993; Hosken et al., 1999; Hellriegel & Bernasconi, 2000). The morphological complexity of the female reproductive tract suggests that some structures may be adaptations designed to influence the storage and usage of sperm from males of different genotypes (Otronen, 1997; Ward, 2000, 2007). Since these female reproductive traits are likely to exert selection on male reproductive traits, the morphology of the female reproductive tract may be an important determinant of male reproductive success. Although there is strong comparative evidence that female reproductive traits (the spermathecal duct) and sperm length coevolve (Minder et al., 2005), the underlying mechanisms acting within species are still largely unknown.

To indirectly examine reproductive interactions between male and female yellow dung flies, we set out to estimate the phenotypic and genetic variation underlying a number of male and female reproductive traits, as well as the patterns of co-variance among them. We expected positive phenotypic and genetic correlations for traits within sexes that are functionally integrated and/or linked via resource acquisition (e.g., between accessory gland size and accessory gland duct length, or between spermatheca size and spermathecal duct length; van Noordwijk & De Jong, 1986). In contrast, we expected negative correlations between expensive life history traits that compete for metabolic resources (e.g., accessory glands and spermathecae). Intersexual genetic correlations might be positive if structures share genes that have pleiotropic influences on development in both sexes (e.g. genes for reproductive effort expressed in both sexes, Zajitschek et al., 2007, Falconer, 1981; van Noordwijk & de Jong, 1986, De Laguerie et al., 1991), or if structures interact mechanistically (e.g., the length of sperm and the length of spermathecal ducts; Presgraves et al., 1999); and intersexual genetic correlations may be negative for fitness-related traits if the fitness level of the shared allele is expressed differently in both sexes (e.g. spermatheca size and testis size; Thüler et al., unpublished, chapter 4).

MATERIALS AND METHODS

Fly breeding

In October 2004 we collected 42 pairs of *Scathophaga stercoraria* at Fehraltorf, near Zurich, Switzerland, and reared them in the laboratory using standard procedures for two generations before experiments were carried out (see Ward & Simmons, 1991; Ward, 1993). Larvae were reared on cow dung at 20° C, 66% relative humidity and a 12:12 dark-light regime. After emergence, adult flies were individually kept in glass vials and fed *ad libitum* with water, sugar and live adult *Drosophila melanogaster* as prey. We then paired unrelated males and females randomly. After a pair had mated, the female was allowed to lay eggs into a small amount (c. 2 g) of fresh cow dung. Adults were thereafter killed by freezing and stored individually at -20° C in Eppendorf tubes. We counted the eggs and placed them in plastic containers with enough cow dung (at least 2 g per larva) to prevent larval competition for food (Amano, 1983). Males of the third laboratory generation were randomly mated with three non-sibling females from the same

generation and their adult offspring were again housed individually using the methods above. Placement of the dung containers and the vials containing the adults within the climate chamber was changed regularly to account for potential small scale environmental differences. Note that although the set-up in generation 3 is identical to a traditional half-sib design, we in fact know the exact pedigree relationships between all individuals, both within and across all four generations.

Measurements

We dissected all 3rd generation parents and 4th generation offspring (59 fathers, 172 mothers, 564 sons and 602 daughters) and measured the following female traits (figure 1), using the mean size of all paired organs: the size of all spermathecae (two-dimensional area), the length and diameter of all spermathecal ducts, the size of both accessory glands, and the length of both accessory gland ducts. For males we measured the size of both testes, sperm head length, and total sperm length (five sperm per male). Although sperm total length includes sperm head length, Humphries (2008) found that the relative length of sperm components is more likely to be targeted by selection than the total length; we therefore retained both traits in the analysis. As an index of body size we measured the hind tibia length (HTL) of both males and females (Sigurjónsdóttir, 1980; Ward & Simmons, 1991). We performed the measurements using images conveyed from a binocular stereomicroscope through a microscope mounted camera to a PC running ZEISS KS300 software. We then used linear distances for lengths (including curves) by manually clicking c.50 waypoints along the centre of each traits, and traced the outlines of two-dimensional structures on the PC screen to calculate their area. To measure sperm head length and total sperm length, the testes were dissected in distilled water on a microscope slide. Each testis was pierced in the proximal third (close to the ejaculatory duct) to obtain only mature sperm. The testis was then removed, the sperm gently diluted in the droplet of distilled water and dispersed on a glass slide. After the slide was air-dried, a drop of DAPI (4',6-Diamidino-2-phenylindole) was added to stain the DNA in the sperm head. We obtained fluorescence micrographs of the stained sperm head and light micrographs of the whole sperm. We measured five sperm per individual using the public domain image analysis software ImageJ (available at http://rsb.info.nih.gov/ij/) by manually clicking about 30 waypoints along the centre of each sperm and sperm head, and summing the linear distances between each waypoint.

Statistical analyses

Standard least-square analysis of half-sib breeding experiments only uses the phenotypic resemblance among sibs and half-sibs (Lynch & Walsh, 1998). In this particular case, however, we also have phenotypic data on the parental generation and pedigree information for four generations. To make optimal use of all available data, we therefore chose to estimate the amount of phenotypic (co)variance attributable to additive genetic and maternal effects by means of an animal model and restricted maximum likelihood (REML) estimation procedures, implemented in ASReml (Gilmour et al., 1998). The animal model is a form of mixed effects model that is able to simultaneously use data on more than one generation (Lynch & Walsh, 1998; Kruuk, 2004). By using all relationships between individuals in a

pedigree, an animal model typically provides considerably more estimation power than conventional methods (e.g. ANOVA, Lynch & Walsh, 1998). Moreover, it allows for the simultaneous inclusion of additional random and fixed effects, and it is better able to deal with unbalanced data sets (Shaw, 1987; Knott et al., 1995). Phenotypic correlations were estimated using SPSS for Windows (version 14).

We first estimated heritabilities using a multivariate model that included all ten traits, but with the co-variances for the animal, the mother and the residual term all fixed to zero (which is similar to running ten separate univariate analyses). The inclusion of maternal identity in the model as a random effect accounted for the resemblance among offspring from the same mother due to maternal and non-additive (dominance) genetic effects, allowing us to obtain more accurate estimates of the additive genetic variance V_A. Significance of V_A was determined using a series of likelihood ratio tests, testing whether fixing V_A to zero for a trait resulted in a significantly worse fit of the model. We present one-sided P-values, as we test the hypothesis $h^2>0$, not $h^2<>0$ (see e.g. Fry, 2004). Coefficients of additive genetic variation were included for completeness (Houle, 1992). Second, we derived a series of bivariate models, both within and across sexes. When estimating the between-sex genetic correlations, the residual co-variance is meaningless and cannot be estimated, and was therefore fixed to zero. These models provide us with an estimate of all pair-wise genetic correlations and their approximate standard errors. Significance testing was again performed using likelihood ratio tests, comparing models that estimate additive genetic variances as well as the genetic correlation between the traits to models in which the correlation is fixed to zero (two-sided tests). Additionally, we tested whether genetic correlations are significantly smaller than 1, or significantly larger than -1 (again using one-sided tests).

Some of the matings took place between related animals, creating non-negligible levels of inbreeding. By tracking genetic information throughout the whole pedigree, the animal model (unlike a standard mixed model analysis fitting sire and dam nested within sire) accounts for the problems posed by inbreeding (assuming that the base population consists of unrelated, outbred individuals). However, it does not account for the potential existence of inbreeding depression. Because there was at least some evidence for inbreeding depression in a couple of traits (e.g., sperm length, female body size), as well as for an interaction between generation and inbreeding (e.g., in female body size) we account for any potential effects of inbreeding by including the inbreeding coefficient (F) and its interaction with generation in all models, irrespective of whether there was significant inbreeding on the trait. Note that we included F in our models to account for any additional (residual) variation inbreeding may introduce, although within the context of this study we are not particularly interested in the effect of inbreeding itself. Including F as either a continuous or categorical variable produced similar values, but fitting F as a covariate is more straightforward. Furthermore, although we corrected for F in all models irrespective of whether there was no statistically significant effect, estimates from models that do not fit F and F x Generation are in fact quantitatively very similar.

RESULTS

Heritabilities and trait values

All traits except female and male body size and sperm total length were significantly heritable (table 1). Coefficients of additive genetic variation (CV_A) were high for spermatheca size, accessory gland size and duct length, moderately high for spermatheca duct length, male body size (HTL) and testis size, but relatively low for female body size, spermathecal duct diameter, sperm total length and sperm head length.

Phenotypic correlations

Within females, we found several highly significant positive phenotypic correlations between traits (table 2, above the diagonal), and significantly negative phenotypic correlations between spermathecal duct diameter and accessory gland size, and between spermathecal duct diameter and spermathecal duct length. In males, we found a significantly positive phenotypic correlation between male body size and testis size.

Genetic correlations within sexes

We also found a number of highly significant genetic correlations within the sexes. In females there was a strong positive genetic correlation between accessory gland size and accessory gland duct length, and a strong negative genetic correlation between female hind tibia length and accessory gland size (table 2). Most of the non-significant genetic correlations are negative.

None of the genetic correlations between any of the male traits were significantly different from zero, but all were negative, except for the unsurprisingly positive correlation between sperm head length and total sperm length. The genetic correlations between male HTL and testis size and between male HTL and sperm length were significantly smaller than 1.

Genetic correlations between sexes

Across the sexes, we found significant negative genetic correlations between male HTL and spermatheca size, between male HTL and accessory gland size, and between testis size and spermatheca size. There were no consistent patterns of co-variance among the non-significant intersexual correlations, with 10 values being positive and 11 being negative (table 2).

DISCUSSION

Within the sexes phenotypic, and sometimes genetic, correlations were primarily positive if they were not nil, suggesting functional integration between traits or condition dependent investment in traits. Negative correlations, potentially suggesting resource allocation trade-offs, were only evident between female spermathecal duct diameter and both accessory gland size and accessory gland duct length. Interestingly, some intersexual genetic correlations were strongly negative, primarily those between male body size and both spermatheca size and accessory gland size, and that between testis size and spermatheca size. These

correlations are consistent with the presence of intersexual (ontogenetic) conflict between these structures mediated by sexual conflict over the control of sperm storage and use, although several alternative explanations could also account for this co-variance. We discuss these results in more detail below.

Genetic correlations between the sexes

Arguably the most interesting result of this study is the significant negative intersexual genetic correlation between testis size and spermatheca size, which suggests that females with genes for larger spermathecae tend to have male relatives with genes for smaller testes and *vice versa*. In addition, both spermatheca and accessory gland size are negatively genetically correlated with male body size (hind tibia length, HTL; table 2). Previous research has shown for this species and insects in general that larger females have a fitness (fecundity) advantage as they lay more eggs (Jann et al., 2000; Honek, 1993). These females also tend to have larger spermathecae (this study). One might therefore speculate that larger females might favour males with larger testes, since these males have more sperm to fill the female spermathecae, and therefore may have an advantage in sperm competition. However, assortative mating by size has never been found in the field (Jann et al., 2000) and such assortative insemination success should lead to positive co-variance between spermathecal size and testis size (Morrow & Gage, 2000; Minder et al., 2005); here we found the opposite. One possible explanation is that these two traits (or unmeasured traits closely linked to them) covary positively with fitness and are involved in an intralocus sexual conflict, (Chippindale et al., 2001; Zajitschek et al., 2007), which may result in an intersexual negative genetic correlation for fitness-related traits (such as male testis size and female spermatheca size, see introduction). This suggests that both traits are affected by the same loci and although selection on female traits may result in adaptive evolution of male traits, both sexes may be selected toward a different phenotypic optima (Rice, 1984; Lande, 1987; Parker & Partridge, 1998; Chippindale et al. 2001).

Negative genetic correlations can also result from coincidentally strong directional selection on both traits (Falconer & Mackay, 1996; Roff, 1997; Jensen et al., 2003).). This is because genetic covariation aligned with the main direction of selection will become depleted, leaving most of the genetic covariation oriented orthogonally to the main axis of selection (Hine & Blows, 2006; Roff, 1997). Simultaneous selection for both traits could have depleted the positive genetic co-variance that had existed within the traits, leaving only negative genetic co-variance to be discovered. For example, early and late fecundity are positive correlated, but after selection and fixation, at equilibrium the only loci that will still be segregating are those producing an increase in one fitness-related trait, but a decrease in the other (Hazel, 1943; Roff, 1997).

Intersexual genetic associations between traits can indicate correlated evolution of male and female reproductive structures (Minder et al., 2005). In several studies with insects, covariation between female reproductive characters and sperm length has been documented. For example, there is positive co-variance between sperm length and the length of the female spermathecal ducts both across species (e.g., in stalk-eyed flies and scathophagid flies; Presgraves et al., 1999, Minder et al., 2005) and within species across replicate selection lines (in *Drosophila melanogaster*, Miller & Pitnick, 2002). Our estimate of the

genetic correlation between spermathecal duct length and sperm length was also positive, although non-significant. If this non-significant positive co-variance is real (and our failure to detect it was only a problem of statistical power), it seems plausible that it could arise via correlated evolution. For example, extending the spermathecal duct length may be one instrument by which females gain more control over sperm storage, and males with longer sperm may be better able to achieve insemination within females with longer spermathecal ducts.

Another interesting pattern was the non-significantly positive genetic correlation between female accessory gland size and testis size. Accessory glands contain a fluid that may affect sperm viability, either by killing sperm (Hosken & Ward, 1999; Bernasconi et al., 2002) or nourishing them (Thüler et al., unpublished, chapter 4). It could be that males with large testes are able to produce more sperm, making them better at resisting the sperm viability manipulations of female accessory gland secretions. More work documenting the consequences of these glands for sperm competition are needed, however, before the conflicting evidence present can be understood (Hellrigel & Ward, 1998; Bernasconi et al., 2002; Thüler et al., unpublished, chapter 4). Nevertheless, the positive correlation found here may suggest that accessory gland and testes could be engaged in an arms race (Pischedda & Chippindale, 2006; Simmons & Siva-Jothy, 1998; reviewed in Gillott, 2003), perhaps involving control over the fate of sperm in storage.

Correlations within the sexes

Within-sex phenotypic correlations were significantly positive across many traits that seem unlikely to be directly functionally linked, such as body size (HTL) and spermatheca size or HTL and spermatheca duct length in females, and HTL and testis size in males. The genetic correlations were often similar in magnitude to the phenotypic correlations, and almost always of the same sign. Such positive correlations may indicate that many of the measured traits exhibit condition dependence, as individuals that acquire more resources can allocate more across many traits simultaneously. The strongest positive correlations occurred between traits that are also functionally associated, such as between spermatheca size and the length and diameter of spermathecal ducts or between accessory gland size and accessory gland duct length. The genetic correlation between accessory gland size and accessory gland duct length was also highly significantly positive. Where we failed to find statistically supportable phenotypic relationships among traits (i.e. correlations of zero), which we found reasonably commonly (table 1), we can cautiously conclude that the traits are not functionally integrated or may not be sufficiently affected by resource acquisition for a correlation to have been revealed in our experiment.

We also found a significant and strongly negative genetic intrasexual correlation between female HTL and accessory gland size. We can suggest at least two plausible alternative explanations: (1) both traits may have been under persistent selection in the same direction, which has depleted standing genetic positive co-variance (and therefore only negative co-variance remains to be detected; Falconer & Mackay, 1996; Roff, 1997; Jensen et al., 2003; cf. above) or (2) the correlation results from a trade-off in resource allocation. However, while a negative genetic correlation may imply a trade-off, it is worth noting that we see such a pattern for no other pairs of traits. It is hard to imagine that HTL and accessory glands are

particularly expensive traits to build and maintain, justifying a careful balance in investment for these two traits that is lacking across other characters. Further research is needed to disentangle the functional and energetic relationships among these traits.

Heritabilities

We found significant heritabilities for the size of most reproductive tract characters in both females (spermathecae, spermathecal ducts, accessory glands and accessory gland ducts) and males (testis size and sperm head length, but not the total length of sperm; table 1). The lack of heritable variation in sperm length contradicts Ward's (2000) demonstration of high heritability ($h^2 = 0.67$) for this trait. Another inconsistency with other studies is that female and male body size (hind tibia length, HTL) showed very low and non-significant heritabilities, whereas Blanckenhorn (2002) and Teuschl et al., (2007) reported relatively low but significant heritabilities for HTL in yellow dung flies (around $h^2 = 0.35$). Furthermore, while we found high phenotypic variation in spermathecal duct length, accessory gland size, accessory gland duct length, and testis size, phenotypic variation in HTL was very low in our experiment, potentially relating to the highly standardized rearing methods in the laboratory.

Since the strength and sign of a correlation can provide insight into whether and how the involved structures exert selection on members of the opposite sex, we can conclude that any negative or positive inter- and intra sexual genetic correlation (such as the negative correlation between testis size and spermatheca size found here) indicates at least the potential for these traits to coevolve. Moreover, this negative intersexual genetic correlation may even be indicative of intersexual ontogenetic conflict between these structures, which can be mediated by sexual conflict over the control of sperm storage and use. Further research is needed that might distinguish between these alternatives and conclusively demonstrate any intersexual conflict and to measure the magnitude of any influence of sexual conflict on fitness in yellow dung flies of both sexes. One possibility avenue for further study involves the direct manipulation of reproductive traits in either sex, for example by artificial selection, to observe correlated responses of other traits in both sexes. Such a study might confirm the genetic relationships described above. In the next chapter (Thüler et al., unpublished, chapter 2) we artificially select on spermatheca number, with an explicit goal being the documentation of correlated responses to this selection. We argue that the shift from three spermathecae to four spermathecae represents a significant jump in complexity that is naturally variable and probably affects on sperm sorting (Ward, 2000). However, this complexity is also clearly associated with energetic and structural costs, and the balance of costs and benefits should play a large role in determining the optimal female investment in mate choice. Thereafter, by manipulating larval diets (Thüler et al., unpublished, chapter 3) we can study how investment in costly choice apparatus may change with the levels of resource acquisition.

ACKNOWLEDGEMENTS

We thank Julia Burgener, Fabienne Di Gennaro, Sandra Gartner, Rahel Leugger, Barbara Schnüriger, Manuela Varini and Ursula Briegel for their help with the flies and in the lab. We also thank Andy & Katharine Pemberton for discussions and constructive comments on the experiment.

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FIGURES AND TABLES

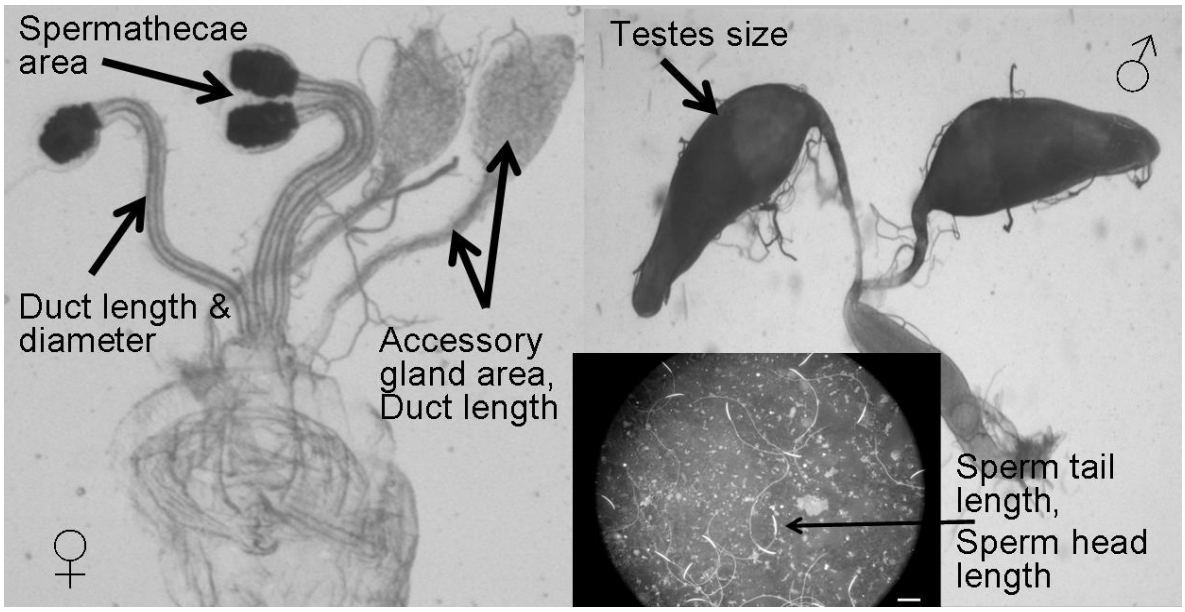

Figure 1. Left: female genital tract with spermathecae area/size, spermathecae duct length and diameter, accessory gland area/size, accessory gland duct length; right: male genital tract with testis size, sperm head and sperm total length.

Table 1. Trait values, variance components and heritabilities for all female and male traits (significant values are in bold).

Trait (unit)	Mean	V_P	V_M	V_A (SE)	V_R	h^2 (SE)	*P-Value*	CV_A
Female Traits								
Female Body Size (Hind Tibia Length, mm)	4.29	5.02	1.94	0.10 (0.38)	2.98	0.02 (0.08)	0.42	0.10
Spermatheca Size (mm^2)	0.11	57.51	6.01	19.31 (5.77)	32.19	0.34 (0.09)	**<0.01**	19.58
Spermathecal Duct Length(mm)	0.74	7881.00	0.00	2438.39 (692.70)	5442.31	0.31 (0.08)	**<0.01**	2388.54
Spermathecal Duct Diameter (mm)	0.01	3.38	0.76	0.93 (0.43)	1.69	0.27 (0.12)	**0.04**	0.90
Accessory Gland Size (mm^2)	0.27	3141.53	524.39	1015.15 (336.60)	1601.99	0.32 (0.10)	**<0.01**	1039.29
Accessory Gland Duct Length (mm)	0.60	12200.13	2349.50	1492.12 (1080.00)	8358.51	0.12 (0.09)	**0.03**	1617.77
Male Traits								
Male Body Size (Hind Tibia Length, mm)	5.29	8.53	3.09	0.93 (0.80)	4.51	0.11 (0.09)	0.08	0.88
Testis size (mm^2)	0.91	6202.14	1790.54	1044.71 (586.30)	3367.16	0.17 (0.09)	**<0.01**	1167.41
Sperm Total Length (um)	212.15	902.54	159.88	44.39 (68.68)	698.27	0.05 (0.08)	0.34	28.90
Sperm Head Length (um)	28.57	20.06	0.45	4.14 (2.12)	15.47	0.21 (0.10)	**0.01**	4.01

(V_P: phenotypic variance, V_M: maternal variance, V_A: additive genetic variance, V_R: residual variance, h2 (SE): narrow sense heritability with standard error, CV_A: coefficient of additive genetic variance; mean: values changed in effective size, for calculations unit values were used)

Table 2. Phenotypic correlations in the upper half, pair-wise genetic correlations (standard errors in brackets) in the lower half of the matrix (significant values in bold).

	Female Body Size (HTL)	Spermatheca Size	Spermatheca Duct Length	Spermatheca Duct Diameter	Accessory Gland Size	Accessory Gland Duct Length	Male Body Size (HTL	Testis size	Sperm Length	Sperm Head Length
Female Body Size (HTL)		**0.27**	**0.098**	**0.26**	0.01	0.01				
Spermatheca Size	-1.00		**0.17**	**0.24**	0.07	0.02				
Spermatheca Duct Length	1.00	-0.13 (0.22)		-0.02	**0.09**	**0.12**				
Spermatheca Duct Diameter	1.00	0.42 (0.25)	0.16 (0.30)		**-0.12**	**-0.09**				
Accessory Gland Size	**-1.00**	-0.08 (0.23)	-0.13 (0.24)	-0.25 (0.27)		**0.41**				
Accessory Gland Duct Length	-1.00	-0.30 (0.31)	-0.28 (0.34)	-0.48 (0.36)	**1.00**					
Male Body Size (HTL	-0.40 (1.78)	**-0.89 (0.52)**	0.02 (0.41)	-0.55 (0.43)	**-0.76 (0.44)**	-0.76 (0.53)		**0.16**	-0.06	0.02
Testis size	-0.72 (0.72)	**-1.00**	-0.14 (0.32)	-0.47 (0.31)	0.45 (0.25)	0.46 (0.40)	-0.22 (0.46)		-0.01	0.06
Sperm Length	-1.00	0.51 (0.97)	0.52 (0.60)	-0.52 (0.81)	-0.35 (0.46)	1.00	-1.00	-0.35 (0.72)		**0.21**
Sperm Head Length	0.69 (0.69)	0.32 (0.27)	0.34 (0.25)	0.46 (0.31)	-0.42 (0.26)	-0.52 (0.33)	-0.17 (0.48)	-0.50 (0.31)	0.36 (0.78)	

Estimates of 0.999 or -0.999 typically occur when VA of one of the two traits is very close to zero and the estimate of the correlation gets bounded at the upper or lower limit of the correlation coefficient. In these cases no standard error can be obtained. We can however still test for their significance using LRT (i.e. the models do converge). Unless stated otherwise, these estimates are not significantly different from zero.

CORRELATED RESPONSES TO SELECTION ON A REPRODUCTIVE TRAIT AFFECTING CRYPTIC FEMALE CHOICE IN YELLOW DUNG FLIES (*Scathophaga stercoraria*)

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ABSTRACT

Pre- and post-copulatory female mate choice may influence the evolution of reproductive traits and the outcome of paternity in many species. Female yellow dung flies have control over sperm storage in part because they have multiple spermathecae. Previous studies have demonstrated that females with four spermathecae are better able to influence paternity than those with only three. Most females from the field have three spermathecae. In our study population around 2% of wild females have four. This represents a substantial jump in complexity of the sperm storage apparatus that is naturally variable and may have a clear impact on sperm sorting ability. We selected on spermatheca number in replicate lines of yellow dung flies to investigate whether building and maintaining such a complex reproductive tract incurs substantial cost for females. We investigate the potential underlying genetic basis of female choice according to spermatheca investment and the genetic trade-offs with other traits. Further we examined correlated responses to this selection in female and male traits and analysed potential costs for females. We successfully increased the fraction of females with four spermathecae to about 50% after eight generations of selection. Although the total sperm storage volume (total spermatheca volume) for females with expressed four spermathecae is effectively larger than females with three spermathecae, we found a trade-off between spermatheca size and number, representing a possible cost for complex reproductive traits. Further, we found that females in 4-spermatheca-lines laid significantly larger clutches than females in 3-spermatheca-lines. In contrast, clutch size decreased with increased spermathecae size, representing a possible trade-off in resource allocation. Furthermore, in 4-spmerathecae-lines females also have larger accessory glands and males larger testes. If a fourth spermatheca indeed allows more effective sperm sorting and therefore more female control over paternity the relationships between investment in important choice structures (spermathecae) and other reproductive traits in females and males will have consequences for post-copulatory sexual selection and implications for sexual conflict.

INTRODUCTION

Darwin (1871) explained that many elaborate male ornaments might arise because of female mating preferences. Bakker & Pomiankowski (1995) showed further that female mate preferences evolve adaptively and these are a major cause of variation in male sexual traits (Gavrilets et al., 2001). Although sexual selection predicts that both males and females should gain benefits by choosing their mating partners, the different sexes may have different criteria for choosing their mates (Lihoreau et al., 2008). Female and male mate choice may occur prior to, during and after copulation and both sexes may influence the outcome of paternity of subsequently produced offspring and the evolution of the reproductive traits in the other sex. For example, competition between males leads to intersexual selection affecting male traits that increase a male's chance to access females (Holland & Rice, 1999). Eberhard (1996) suggested that females are even more likely to affect the outcome of male-male competition after than before mating, because most post-copulatory competition for paternity between males (i.e. male's sperm) takes place within the female's reproductive tract. Therefore, female morphology and physiology may often influence male success in these contests (Eberhard, 1996). For example, the complex reproductive tract of female yellow dung flies may interact with sperm morphology to create a sperm storage pattern to separate favoured and disfavoured sperm separately to a certain extent in her sperm storage organs (spermathecae; Otronen et al., 1997).

Females may influence paternity in many different ways by cryptic mate choice. One way is by changing the duration of mating and therefore the number of sperm transferred. For example, female hanging flies allow males that provide larger nuptial gifts to copulate longer and therefore to transfer more sperm. If the gift is inadequate the female can terminate copulation before insemination is completed (Thornhill, 1976). Female field crickets also manipulate insemination success by adjusting the attachment time of their mates' spermatophores depending on their attractiveness (Sakaluk 1984; Simmons, 1986; Sakaluk & Eggert, 1996; Garcia-Gonzalez & Simmons 2005). Another way is the cryptic *sperm* choice by females (Birkhead, 1998). For example, the variation of larval performance and pupal mass in yellow dung flies depends on male genotype by temperature interactions (Ward, 1998, 2000). Females use sperm from males with suitable genotypes for the relevant environment to increase offspring fitness (Ward et al. 2002). Offspring fathered by males with the suitable genotype may perform better in a certain environmental condition. Not all cases featuring female effects on the outcome of sperm competition are examples of direct female manipulation; in some cases, the female influence is indirect. Otronen & Siva-Jothy (1991) showed that males of the fly *Dryomyza anilis* force a female to release a droplet of stored sperm of previous matings by tapping her external genitalia with his genital claspers. The more tapping a male engages in, the more likely his sperm will fertilize her eggs. Female choice is indirect in this example because although females may resist the effect of this tapping, larger and stronger males are more likely to achieve high levels of insemination. Further, females (e.g. *D. anilis*) can influence paternity by sorting the sperm of their mates. Otronen (1997) showed that the different sperm storage organs of these flies may

have separate functions during sperm storage and use. Famales favored sperm stored in the singlet spermatheca over sperm stored in the doublet spermatheca or in the bursa copulatrix.

Study organism

Complex female reproductive tracts – and therefore complex choice apparatus – are common in insects. The yellow dung fly, *Scathophaga stercoraria,* is an important study organism used as a model organism for sperm competition (Parker, 1970a) and cryptic female choice (Eberhard, 1996) and because females have such complex reproductive tracts. They usually contains three spermathecae, each with a duct leading to the bursa copulatrix (Hosken, 1999; Thüler et al., unpublished, chapter 1, figure 1). The fact that spermatheca number is heritable makes this system amenable to study via artificial selection. Ward (1993) suggested that the complex reproductive tract of female yellow dung flies evolved to provide females more control over sperm storage and use (Ward et al., 2007). Therefore, this system may include a more tangible and potentially quantifiable kind of "mate choice investment" than many other systems. The construction and maintenance of such a complex reproductive tract represents a substantial cost for females (Ward, 2000; Ward et al., 2007). Ward et al. (2007) showed that females with a fourth spermatheca laid significantly smaller clutches, but there may be other costs related to the complex female reproductive tracts, for example, involving genetic correlations to other traits in males and females (e.g., body size, spermatheca size, testis size).

Genetic correlations may occur between investments in a trait that enables more female choice and other life-history and morphology traits in males and females. In a selection experiment on spermatheca number Ward et al. (2007) showed that within 4-spermathecae-lines (females selected up to four spermathecae), the fertilization success of a female's second mate (P2) was significantly lower for larger females, that laid larger clutches, than for smaller females, that laid smaller clutches. Females in 3-spermathecae-lines showed similar P2 values regardless of female size. Since there is a significant positive phenotypic correlation between female body size and spermatheca size (Thüler et al. unpublished, chapter 1) these results suggest that large females may benefit from increased spermatheca number by being able to act against male interests of maximizing paternity (Ward et al., 2007).

Aim of the study

Ward (2000) and Ward et al. (2007) have shown that it is possible to artificially select for increased spermatheca number in yellow dung flies. After 15 generations, replicate lines selected up and down for spermatheca number produced only females with four or three spermatheae, respectively. Unfortunately, because of the labour-intensive requirements for keeping these lines, they were destroyed following the initial studies before a full characterization of the correlated responses to selection on spermatheca number could be completed.

We repeated Ward's experiment (Ward, 2000; Ward et al., 2007) and selected for a trait that is arguably strongly involved in cryptic choice. We examined genetic relationships between spermatheca number and other phenotypic traits in both sexes and investigated the effect of changing the female

morphology on other traits in males and females. But the experiment is only good at finding male traits that are physically linked to genes for female preference, as the genetic correlations between female and male traits (e.g. spermatheca size and testis size) found in chapter 1 (Thüler et al., unpublished). As we only selected on females, we therefore expect a correlated response in males (Blanckenhorn, 1998, 2002), for example between spermatheca number/size and testis size since large testes provide more sperm and males with large testes may perform better within females with more/large spermathecae.

MATERIALS AND METHODS

Selection lines, experimental flies and measurements

We collected sixty-five pairs of yellow dung flies at Fehraltorf (near Zürich, Switzerland) in November 2006 and brought them to the laboratory. After the females laid eggs we dissected them and determined their spermatheca number and type (figure 2; according to Ward et al., 2007; characterising see further down). We then established selection lines by assigning emerging flies to one of two regimes depending on whether they had three or four spermathecae (only exact three globular spermathecae were counted as "three"; a heart-shaped and more divided were counted as "four"). After one generation we divided the subsequent generation in each treatment into three replicate lines (for a total of six lines). Adult flies of each generation were maintained individually in the laboratory under standard conditions (see Ward 2000; Thüler et al., unpublished, chapter 1). For subsequent generations we randomly paired males and females within the replicate lines, avoiding sibling matings. We only used offspring of females with the appropriate number of spermathecae to set up the next generation. We dissected males and females from the eighth generation and took pictures of their genital tracts with a microscope mounted camera. We measured female (spermatheca area/volume, spermatheca duct length, accessory gland area, accessory gland duct length, all mean values) and male traits (testis area, sperm head length and total sperm length, all mean values) and used the public domain image analysis software ImageJ (Image Analysis Software ImageJ, http://rsb.info.nih.gov/ij/) for all traits (figure 1). For measuring sperm head and total length, we dissected a testis, released sperm in a drop of distilled water on a microscope slide and, after air-drying, we added a drop of DAPI (4',6-Diamidino-2-phenylindole) to stain the DNA in the sperm head. we took pictures of these stains using a fluorescence microscope (Leica, Mannheim, Germany). As an index of body size we measured hind tibia length (Sigurjónsdóttir 1980; Ward and Simmons 1991).

Characterizing spermathecae types and realized heritability

We defined the intermediate stages in the evolution from three to four spermathecae to a certain spermathecae type (see figure 2; see also Ward et al. 2007): 3s: three globular spermathecae; A: heart-shaped, B: half-separated spermathecae, C: fully separated spermathecae but joint duct, D: ducts start to separate, E: upper half of the ducts is separated, F: only half of the lower duct is still joint, G: fully separated spermathecae and ducts. Further, we estimated the realized heritability of effectively expressed

four spermathecae in 4-spermathecae-lines over 6 generations (flies under selection) by calculating the ratio R/S (*R*–the response to the upward selection, *S*–the selection differential; Falconer, 1989).

Statistical Analyses – correlated response / GLM

We used a GLM (SPSS ver. 14; SPSS, Inc., Chicago, IL) approach with selection lines, replicates within selection lines and generation as fixed factors to examine the effects of selection and to analyse correlated response in morphological and life-history traits.

RESULTS

Response to selection – spermatheca types, numbers over generations and realized heritability

The transition from three to four spermathecae occurs in stages: within the four-spermatheca lines we observed many females with three globular spermathecae, then in subsequent generations the singlet spermatheca (singlet spermathecal unit; Otronen, 1997) tended to become heart-shaped or subdivided. In still later generations, we saw a fully separated pair of spermathecae that shared a single spermathecal duct, and finally some female expressed four spermathecae and four fully separated ducts (figure 2 and 3; see also Ward et al. 2007).

Our selection program was successful: in the field the percentage of females (wild flies) with four spermathecae was only 2% (c.f. 10% in Ward, 2000). In our experiment this percentage climbed to 50% after eight generations of selection for higher spermatheca number (figure 5). In the selection for lower spermatheca number the percentage of four spermathecae in 3-spermathecae-lines decreased to 1% in the eighth generation.

The estimated realized heritability of effectively expressed four spermathecae in 4-spermathecae-lines over 6 generations was significantly different from zero ($h^2 = 0.6373$; $p<0.001$).

Response to selection - comparing selection lines

We found that females in 3-spermathecae-lines had larger spermathecae than females in 4-spermathecae lines. This is true for size and volume of the spermathecae (mean size and volume; figure 6, A/B). But within 4-spermathecae-lines, if we look at the total spermatheca size and total volume, females expressing four spermathecae have a larger total spermatheca size and total volume and therefore a larger storage space than females expressing only three spermathecae (figure 6, C/D). Further, females in 4-spermathecae-lines laid significantly larger clutches than females in 3-spermathecae-lines (family means, $F_{1,503} = 15.909$, $P < 0.001$, table 1). This is consistent with clutch size related to spermatheca size and volume over both selection lines: the larger the spermathecae is (size and volume) the smaller is the clutch size. In other words, although females in 3-spermathecae-lines have larger spermathecae, they laid smaller clutches compared to females in 4-spermathecae-lines.

There was no significant difference in longevity between the two lines. Female HTL showed no difference between the lines, but males in 3-spermathecae-lines were larger (51.23 mm) than in 4-spermathecae-lines (48.29 mm).

Correlated responses of other morphological traits between the selection lines are shown in table 1. Females in 3-spermathecae-lines had significant longer spermathecal ducts than females in 4-spermathecae-lines. In contrast, the size of the accessory glands and the length of the accessory gland ducts in 4-spermathecae-lines were significant larger and longer, respectively, than in 3-spermathecae-lines.

Male traits showed the following results: males from 4-spermatheca lines had longer sperm heads than flies in lines selected for 3-spermathecae, but the total sperm length showed the opposite picture: sperm were longer in 3-spermatheca-lines. Finally, males in 4-spermatheca-lines had slightly larger testes than males in 3-spermatheca-lines (table 1).

DISCUSSION

Although very few wild female yellow dung flies have four spermathecae, 50% of the females had this phenotype after eight generations of artificial selection for increased spermatheca number (figure 5). The 3- and 4-spermathecae-lines showed a clear deviation from each other in the direction expected as a result of selection. In 3-spermathecae-lines the percentage of females with four spermathecae decreased to 1% after eight generations of selection. This showed that the variation is partially genetic and it is consistent with the results in Ward (2000), although the realized heritability in our experiment was higher than in Ward et al. (2007) for the expression of effective four spermathecae (phenotype). When heritability for a trait is high, phenotypic selection will be more effective and more response was obtained in the *high* line. The relatively high realized heritability indicates the presence of an additive genetic component in the trait (Toro & Newkirk, 1991).

Interesting differences between the two lines are the mean size/volume of the spermathecae and the length of the spermathecal ducts, which decreased in females in 4-spermathecae-lines, compared to those females in 3-spermathecae-lines (the size of spermathecae in the 3-spermathecae-lines was consistent in every generation; figure 6). Although females in the 4-spermathecae lines are able to build a fourth spermatheca, the mean size and mean volume of each of these organs are significantly smaller than in the 3-spermathecae-lines. This result shows a possible cost of a more complex reproductive tract, probably via a trade-off between number and size/volume of spermathecae. But if we look at the overall (total) size and volume of spermathecae of females with expressed four spermathecae (within 4-spermathecae-lines), they are effectively larger and they therefore have a larger overall storage space than females with the usual three spermathecae (within 4-spermathecae-lines).

One of the most interesting results we found was the negative correlation between spermatheca size and clutch size (figure 7). Although females in 4-spermathecae-lines laid significantly larger clutches than females in 3-spermathecae-lines, if we look at the effective size (mean) of the spermathecae, females

with large spermathecae laid significantly smaller clutches than females with small spermathecae. This represents a cost that may arise through a trade-off in resource allocation between storage space and clutch size.

Although there is a heritable variation in spermatheca number, there are probably other factors involved in the expression of the trait (e.g., condition, which we will discuss in the next chapter; Thüler et al., unpublished), since the percentage of females that have four spermathecae in offspring of wild flies (eggs laid in the lab) increased to 25% after a single generation (4-spermathecae-lines; figure 5) in the lab. It might thus be helpful to experimentally investigate individuals under stress (Blanckenhorn, 1998) by manipulating the larval diet since costs of mating have major impacts in evolutionary models of mate preferences (Pomiankowski, 1987; Kokko et al., 2002).

Several other traits showed a correlated response to selection on spermatheca number: male body size decreased in 4-spermathecae-lines, whereas female body size did not show a correlated response to selection. Interestingly, accessory gland size and accessory gland duct length behave differently than the spermathecae; they become larger and longer, respectively, in 4-spermathecae-lines. Accessory glands contain a fluid that is apparently involved in fertilization (Leopold & Degrugillier, 1973; Leopold et al., 1978) and thought to affect sperm viability in the female reproductive tract (Thüler et al., unpublished, chapter 4). Females may use this fluid to create a hostile insemination site for male sperm or to keep sperm alive, and therefore to potentially facilitate cryptic female choice (Birkhead et al., 1993; Hellriegel & Ward, 1998; Hosken et al., 2001). This would partly explain the larger size of the accessory glands in females in 4-spermathecae-lines, where the females in the 4-spermathecae-lines have a larger overall storage space. Another reason for larger accessory glands in 4-spermathecae-lines could simply be the increased ability of females to acquire and allocate resources. Furthermore, like accessory glands, male testes showed a correlated response to selection such that in 4-spermathecae-lines, males have larger testes. With the above assumption of keeping sperm alive with accessory gland fluid in the female reproductive tract, a male with large testes would perform better in females with large accessory glands (because more sperm might need more nourishment).

If having four spermathecae allows females to more effectively sort sperm, we might expect that genes encoding the 4-spermathecae phenotype are genetically correlated with traits in males that promote insemination success within choosier females. The only pair of characters for which we have this kind of evidence relates the spermatheca size to testis size, however in that case we have actually found a negative genetic correlation (Thüler et al., unpublished, chapter 1), which may suggest a sexual conflict between the sexes. Ward (2000) showed that large females may benefit from increased spermatheca number and may be able to act against male interests (maximizing offspring number). But the extent to which biased insemination arises via indirect selection for genetic benefits rather than direct selection for food acquisition is not clear (Gwynne 2001; Bussière 2002).

Investment in a trait comes at a cost of investment in other traits leading to energetic trade-offs between these related traits (Van Noordwijk & de Jong, 1986). With a fourth spermathecae a females might be able to influence male traits up to a certain extent to serve her own fitness interests (see also

Hellriegel & Ward, 1998; Ward, 1998). Individuals often gain evolutionary benefit by manipulating others (Hunt & Roberts, 2004). In other words, the relationship between investment in an important choice structure (spermathecae number) and other morphological traits in females and males may have consequences for post-copulatory sexual selection and implications for sexual conflict. It is therefore important to separate the effect of the genetic background (selection lines) from the phenotypic expression of the spermatheca number and to examine its influence on life-history traits (e.g. clutch size).

ACKNOWLEDGEMENTS

Many thanks Ursula Briegel, Bettina Eugster and Samuel Tanner for their help in the laboratory and with the selection lines.

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FIGURES AND TABLES

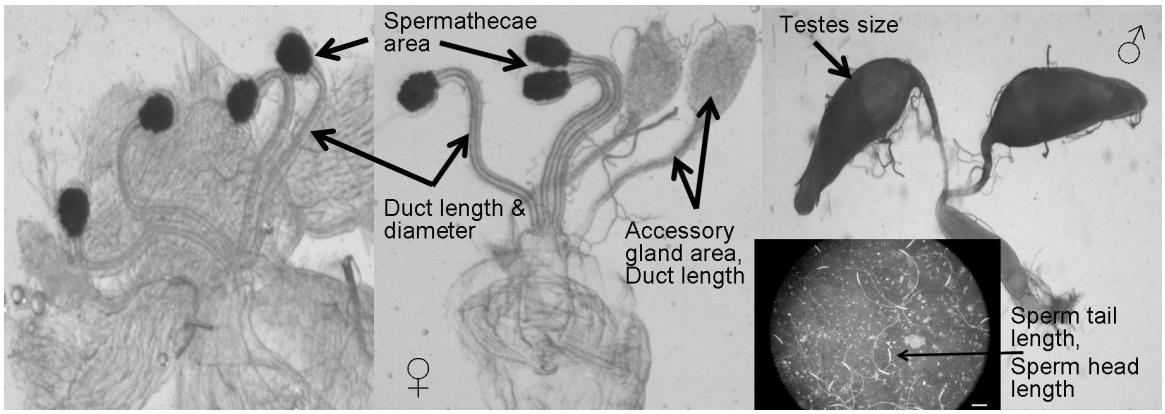


Figure 1. Left and middle: female genital tract with four (left) and three (middle) spermathecae, (spermatheca area, spermathecal duct length & diameter, accessory gland area, accessory gland duct length); right: male genital tract (testis area, sperm head length and sperm tail length).

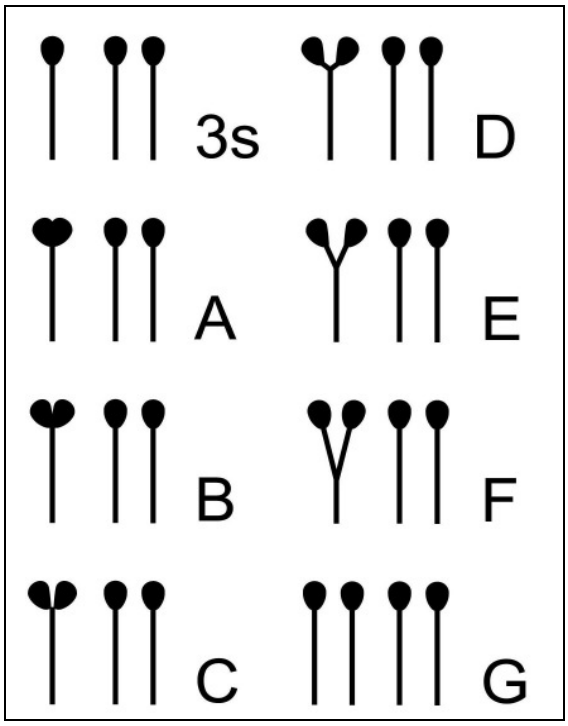


Figure 2. Intermediate stages in the evolution from three to four spermathecae (see also Ward et al. 2007). 3s: three globular spermathecae; A: heart-shaped, B: half-separated spermathecae, C: fully separated spermathecae but joint duct, D: ducts start to separate, E: upper half of the ducts is separated, F: only half of the lower duct is still joint, G: fully separated spermathecae and ducts.

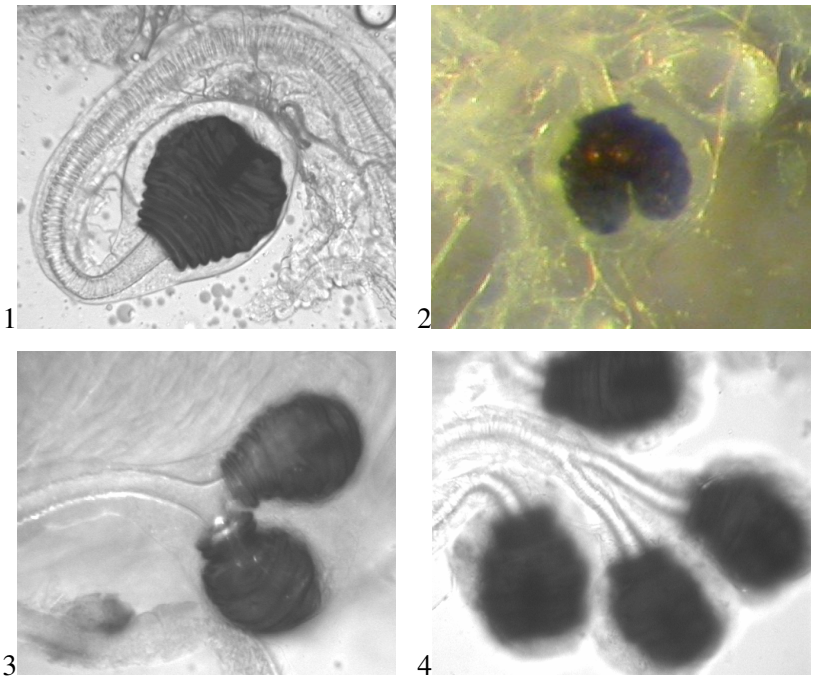


Figure 3. Examples of intermediate stages in the evolution from three to four spermathecae: 1: single globular spermatheca; 2: spermathecae start to separate, type B; 3: spermathecae are fully separated with a joint duct, type D; type F, fully separated spermathecae and a half way separated duct.

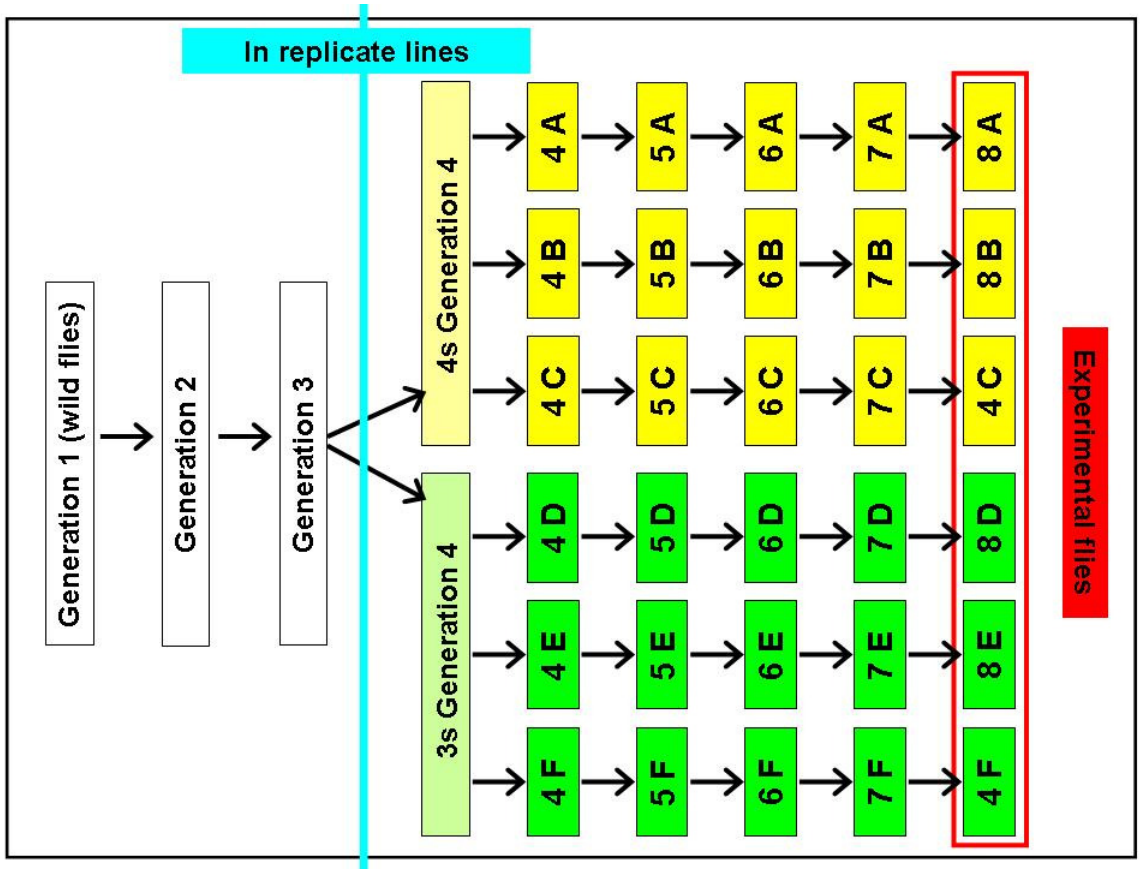


Figure 4. Design of the selection experiment. Adult flies of the third generation were divided in lines of three or four spermathecae and after another generation replicates within each line were set up.

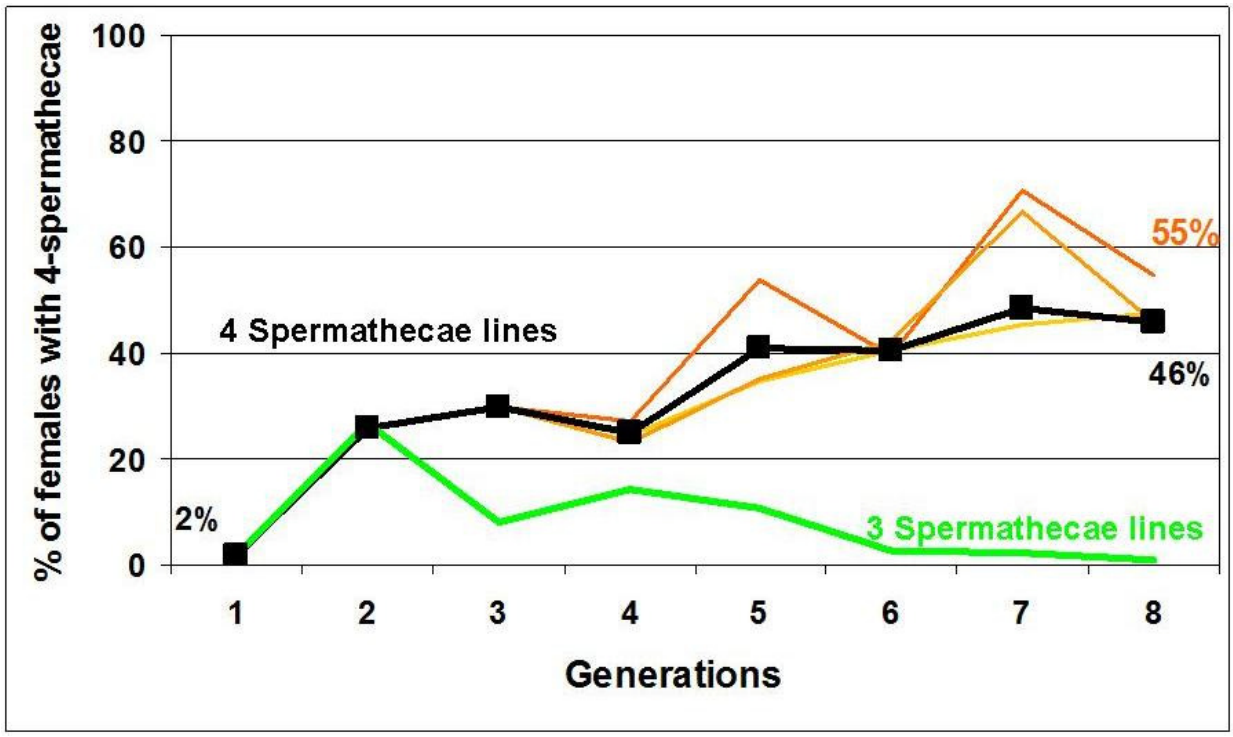
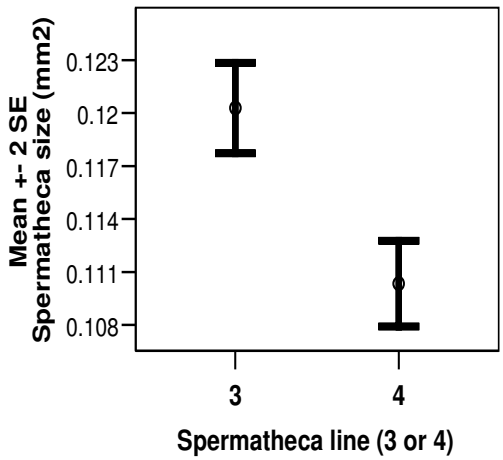
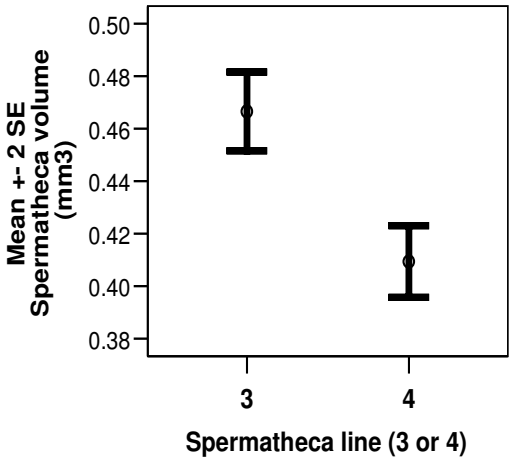


Figure 5: Black line: 4-spermathecae-lines: Percentage of females with four spermathecae increased from 2% in the field to around 50% (54.76% in one replicate). Generation 1: wild flies. Three Orange lines: replicate lines of the 4-spermathecae-selection-line. Green line: 3-spermathecae-lines: Percentage of females with four spermathecae decreased to almost zero.

Mean spermatheca size and volume between selection lines (3 or 4-spemrathecae-lines)

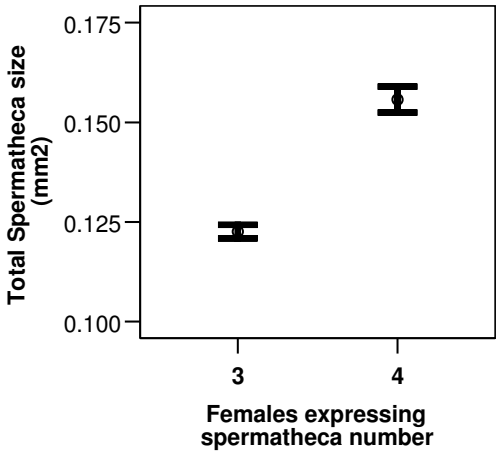


A

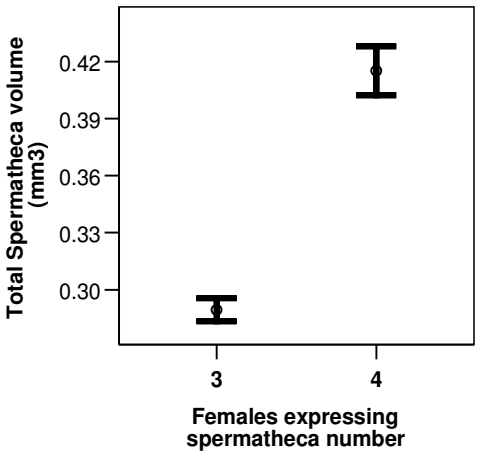


B

Total spermatheca size and volume within 4-spermathecae-lines, between 3 or 4 spermathecae expression

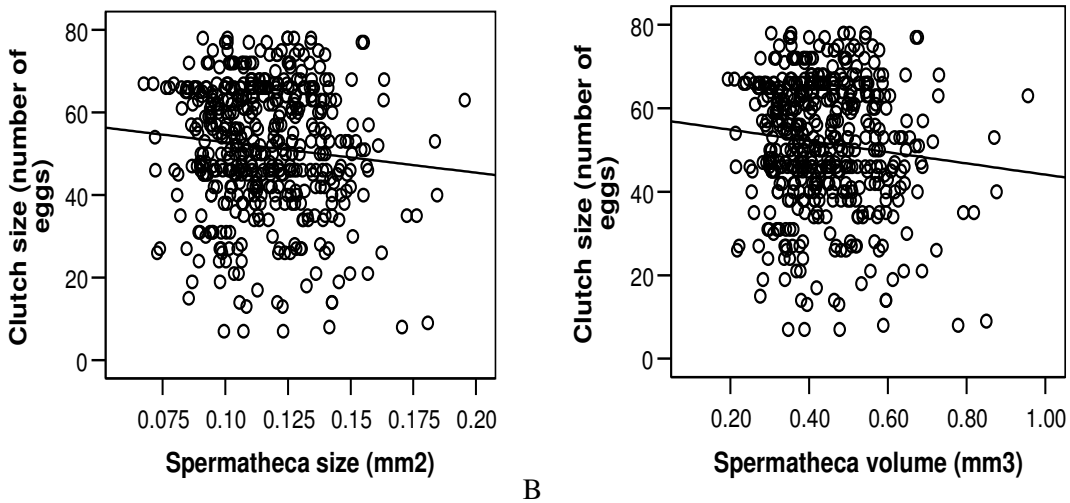


C



D

Figure 6: (A) mean spermatheca size and (B) mean spermatheca volume between 3- and 4-spermathecae-lines; (C) total spermatheca size and (D) total spermatheca volume of expressed three or four spermathecae within 4-spermathecae-lines.



A B

Figure 7: Clutch size (number of eggs) related to spermatheca mean size (A) and mean volume (B).

Table 1. Effect of selection line (left) and replicate nested within selection line (3- and 4-spermathecae lines, right) on various life history and morphological traits as determined by separate GLMs (family means, corrected for body size).

Trait	**d.f.**	**MS**	***F***	***P-Value***	**d.f.**	**MS**	***F***	***P-Value***
life history traits					***Replicates within Selection Lines (nested)***			
Clutch Size (Egg Number)	2	20.055	15.909	**<0.001****	4	504.636	2.168	0.078
Longevity	2	0.016	0.001	0.972				
morphological traits								
Female Body Size (Hind Tibia Length)	1	0.042	0.042	0.838	4	11.665	2.484	**0.048***
Male Body Size (Hind Tibia Length)	1	5.431	5.689	**0.019***	4	177.991	11.146	**<0.001****
Spermatheca size (mean)	2	78.966	114.488	**<0.001****	4	46.517	2.024	0.096
Spermathecae volume (mean)	2	1.081E+012	110.323	**<0.001****	4	5.802E+010	6.143	**<0.001****
Spermathecae Duct Length	2	25.291	27.959	**<0.001****	4	261.196	0.093	0.985
Accessory Glands Size	2	41.537	51.051	**<0.001****	4	831.263	1.360	0.253
Accessory Glands Duct Length	2	14.075	14.828	**<0.001****	4	4623.103	1.627	0.173
Testis size	2	2.529	2.611	**0.079(*)**	4	1452.815	0.968	0.454
Sperm Head Length	2	5.545	6.194	**0.003***	4	6.958	0.637	0.644
Sperm Length	2	3.276	3.505	**0.034**	4	587.275	1.353	0.296

(Significance level p<0.05*; p<0.001**; grey shaded: traits in 4-spermathecae-lines show higher values)

COSTS OF FEMALE CHOICE: CONDITION-DEPENDENCE AND MATERNAL EFFECTS IN SPERM STORAGE ORGAN INVESTMENT IN FEMALE YELLOW DUNG FLIES

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ABSTRACT

Sexual selection may cause the evolution of morphological and reproductive traits. Adaptive female choice may account for some of the diversity in these traits, but discerning the role of post-copulatory female choice on these traits in males and females requires detailed assessments of selection on female that arises because of mating or fertilisation biases. Such assessments will include the possible benefits of mate choice along with detailed measurements of the costs of exercising the choice. For this study, we investigated the costs of investing in a discrete phenotypic structure that is thought to allow postcopulatory mate choice in the yellow dung fly, a model organism for sperm competition and cryptic female choice. Females usually have three sperm storage organs (spermathecae), which are thought to play some role in sorting sperm from different males. To explore the relationship between resource acquisition, investment in post-copulatory mate choice, costly mate choice apparatus (spermathecae) and investment in other aspects of life-history in both males and females we selected up (for the expression of four spermathecae) and down (for the expression of only three spermathecae) on spermatheca number. In the last two generations of this experiment, we also manipulated the quality of dam and offspring larval diet to examine the influence of resource acquisition on investment patterns within and across selection regimes. By statistically separating the effect of the genetic background (selection lines) from the phenotypic expression of spermathecae, we were able to study the cost of investing in spermathecae in terms of its effect on other life history traits. The expression of four spermathecae (in 4-spermathecae-lines) had a significantly negative effect on female fecundity. Further, the spermatheca expression is condition-dependent, because good dam diet influenced the expression of a fourth spermatheca positively (offspring diet had no effect). Neither dam nor offspring diet seems to have any significant effect on male traits. The results support the notion that resource acquisition may be an important factor maintaining genetic variation in costly female choice and thus potentially driving heterogeneity in sexual selection on male sexual traits.

INTRODUCTION

Darwin (1871) explained that sexual selection operates via intrasexual competition or intersexual mate choice. This selection can favour phenotypic variants at both pre- and post-copulatory stages (Birkhead & Møller, 1998; Simmons, 2001). Typically pre-copulatory sexual selection takes the form of, for example, contests among males or mate choice by females (Andersson, 1994). Post-copulatory sexual selection can entail competition among sperm of different males (Parker, 1970) or female influences on male fertilization success. Regardless of whether intrasexual competition occurs before or after mating, it may cause the evolution of morphological and physiological traits (Birkhead & Møller, 1998; Simmons, 2001). In fact, many male reproductive traits (e.g., male genitalia, ejaculate size) have been previously attributed to sperm competition; and any influence of such traits on females was interpreted as an adaptation of males to avoid sperm competition (e.g. mating plugs, substances in the seminal fluid which reduced her desire to re-mate, and mate guarding; Andersson & Simmons, 2006).

However, the relative role of alternative models of post-copulatory selection (i.e., sperm competition versus post-copulatory female choice) on male and female reproductive tract characters remains poorly understood, primarily because of difficulties in measuring and manipulating the effects of these characters on fitness. Evaluating whether adaptive post-copulatory female choice can account for some of the diversity in reproductive tract characters will require more detailed assessments of direct and indirect selection on female traits that cause biases.

Mate choice and its benefits and costs

Although mate choice occurs in males and females, females are generally the choosier sex (presumably because eggs are typically more costly to produce than sperm; Bateman, 1948; Trivers, 1972; Wilson, 1975; Parker, 1979). Mate choice can be active (e.g. in *Drosophila montana,* females actively choose between courting males; Suvanto, 1999) or passive (e.g. in lekking species, like the little bustard *Otis tarda* or the Black Grouse *Tetrao tetrix*), where males fight it out and females mate with the winner; Andersson, 1994), direct (e.g. females select males based on variation in signalling traits, Genner, 2008) or indirect (by generating sperm competition which allows cryptic female choice, because paternity is influenced by the male with high quality sperm; Gage, 2005).

Females may gain direct (e.g. territories, male parental care, absence of contagious parasites) and/or indirect benefits (obtaining good genes from high-quality males) by exercising mate choice (Andersson, 1994; Simmons, 2005) and their choice is often based on the male's phenotypic quality (e.g. costly ornaments, tail length, Andersson, 1994; nuptial feeding, Simmons et al., 1999; bird song repertoire size, Doutrelant et al., 1999; plumage pigments, Dale, 2000; antler Size, Vanpé et al., 2007). Although females can benefit from mate choice, there are potentially structural, metabolic, time and opportunity costs involved with exercising mate choice. Before copulation, female mate choice is influenced by the availability of mating partners (Jennions & Petrie, 1997; Schneider & Bürger, 2006). Choosing a mate is time consuming (Jennions & Petrie, 1997; Ward et al., 2007), and females may experience predation risks

(Pomiankowski, 1987) or may lose energy (Kirkpatrick & Hall, 2004) while exercising choice. For example, visiting territories of active males may impose fitness costs to female marine iguanas, where females lose more body mass during the mate choice period (Vitousek et al., 2007). Further, males may impose direct costs on females during or after copulation: male reproductive traits can sometimes improve male fitness in sperm competition at the expense of female fitness (Stockley, 1997). For example, some accessory gland proteins in the seminal fluid of *Drosophila* are beneficial for males but harmful to females (e.g. by increasing egg laying, decreasing sexual receptivity to other males but simultaneously decreasing female life span and lifetime reproductive success; Arnqvist & Rowe, 2005; Chapman & Davies, 2004; but see Priest et al 2008a, 2008b). Finally, investment in female reproductive anatomy may impose structural costs as seen in insects, where females have complex sperm storage systems, investment in which may trade off with investment in other life history functions such as fecundity (Ward et al., 2007). For example, in yellow dung flies, *Scathophaga stercoraria*, Ward et al. (2007) showed that investment in an extra sperm storage organ with its other associated structures (duct, muscles) cost on average 8% (five eggs) of a female's reproductive output per clutch.

The costs associated with mate choice consist of an importance source of direct selection on female preferences (Pomiankowski, 1987; Kokko et al., 2002, Ward 2007). Insofar as these selection pressures may help reveal the evolutionary basis of complex reproductive tract characters, studying the nature of costs of choice may clarify which of the models of mate choice evolution are chiefly responsible for diversity in these characters (Gavrilets et al., 2001; Eberhard, 1996). Because the balance of costs and benefits plays a large role in determining female investment in mate choice, it is worth considering how this investment may change with different levels of resource acquisition (Hunt et al., 2005).

Condition dependence

Phenotypic condition can be defined as the quantity of metabolic resources acquired by an individual and the efficiency with which these resources are converted into fitness (Bonduriansky & Rowe, 2005). David et al. (2000) found that non-sexual traits (e.g., female eye span, male and female wing length) show genetic variation in condition-dependent expression. Individuals in good condition (e.g., those from optimal environmental conditions with a rich food supply) will generally be able to produce larger sexual traits (Bonduriansky & Rowe, 2005). In female guppies (*Poecilia reticulata*), Syriatowicz and Brooks (2004) showed further that female sexual responsiveness may also be condition-dependent (affected by diet quality). Therefore, investment in choice can similarly depend on condition (Vitousek, 2009) and cause decreased allocation to other important life history traits (Hunt et al., 2005). If there are trade-offs among traits enabling one phenotype to have higher fitness in one environment and another phenotype to have higher fitness in another environment, adaptive plasticity can evolve (DeWitt & Scheiner, 2004). The response of a phenotype to variation in condition is known as phenotypic plasticity, and it plays an important role in the evolution and expression of sexually selected traits.

Phenotypic plasticity can be adaptive or non-adaptive, discrete or continuous (see Bradshaw, 1965; Via, 1984a,b; West-Eberhard, 1989; Windig, 1993) and may have evolved as a result of variable

conditions (Levins 1968; Via & Lande 1985; Sultan & Spencer 2002). Condition dependence represents a form of phenotypic plasticity (Bonduriansky, 2007). A highly plastic trait which strongly depends on environmental conditions (temperature, food availability) is body size (Teuschl et al., 2007). If conditions are good (e.g. high quality food) an individual can invest more than rivals across several life history traits, but growing faster and bigger is energetically, physiologically and ecologically costly (Teuschl et al., 2007).

Often not only the acquisition of an individual itself is an important factor affecting the developmental trajectory, but also maternal provisioning can be relevant. The development of an individual and its traits is influenced by the environment it is living in and the environmental conditions (e.g. food availability or quality). The acquisition of resources important for condition-dependent traits occurs not only during the development of sexual maturity, but also during juvenile or larval development, and often even in early embryonic development. Because mothers provide almost all embryonic provisioning, they can potentially exert a strong influence on condition-dependent sexual traits that are expressed much later in the development of the organism. Environmental effects of mothers on their offspring are called maternal effects (Cheverud & Moore, 1994), and they can have strong influences on offspring fitness. It is therefore important to look closer at these two factors:

Maternal effects are generally defined as a variation in offspring phenotype as a consequence of a mother's phenotype rather than the offspring's own genes (Roff, 1998). Strong maternal effects have been documented to affect offspring development time (e.g., Roff, 1992) and offspring fitness (e.g., Jann & Ward, 1999). For example, in a Neriid fly, offspring of high-condition mothers, which produce larger eggs, have a decreased developmental time even when reared on a poor larval diet (Bonduriansky & Head, 2007). Mothers reared under nutritional stress may have lower offspring fitness (e.g. higher egg mortality, or poorer larval survival) and the maternal nutrition deficiency can affect adult body size (e.g., as shown in yellow dung flies; Jann & Ward 1999). Although maternal effects can have strong influences on offspring fitness they also can act as a buffer for offspring from environmental change or alter the offspring phenotype according to environmental conditions. Mothers may increase provisions per individual offspring by reducing clutch size for example. In other words, a mother may translate her experience into adaptive variation in her offspring (trans-generational phenotypic plasticity; Marshall, 2008).

A comprehensive assessment of costs of choice might involve a plastic aspect of female mate choice, the expression of which depends on resource acquisition. One of the best study systems for this kind of work is the yellow dung fly, *Scathophaga stercoraria*, which is a model organism for investigating sperm competition and cryptic female choice (Ward, 1998).

Biology of the study organism and predictions

Males of the yellow dung fly wait on or around fresh cowpats for females to mate with them. After copulation, females lay their eggs in the fresh cow dung from which the larvae will feed. Although females have no apparent choice of mating partners they do appear to exercise post-copulatory choice by preferentially using sperm from males with desirable genotypes (Ward, 1998; Ward, 2000). Females have

complex reproductive tracts that typically contain three sperm storage organs (spermathecae; Thüler et al., unpublished, see chapter 1 and 2). In the field about 2% of females have four spermathecae. These structures are thought to play some role in sorting sperm from different males (Otronen et al., 19997).

Artificial selection in yellow dung flies has shown that variation among females in spermatheca number is partially genetic (Ward, 2000; Ward, 2007; Thüler et al., unpublished, chapter 2). Producing and maintaining an additional spermatheca is probably quite costly for females. Although Ward et al. (2007) showed in a previous study that females in lines selected for higher spermatheca number suffered a cost of roughly 8% eggs per clutch, the extent to which this cost arises as a consequence of investing in the spermathecal structure (as opposed to other aspects of the genetic background) is not yet clear.

Females with large energy reserves may be better able to allocate resources to costly choice apparatus and therefore to pay the costs of exercising cryptic choice than females in bad condition (Roth & Reinhardt, 2003). The ability to sort sperm could therefore also be condition (i.e. nutrition) dependent (Roth & Reinhardt, 2003). In order to study how the costs of investing in reproductive tract morphology may influence female fitness, we manipulated dam and offspring (larval) diet in experimentally manipulated lines of flies selected in one of two directions for spermatheca number (up to four spermathecae, down to three spermathecae, Thüler et al., unpublished, see Chapter 2). Because our unpublished data suggested that maternal allocation might influence the expression of spermatheca number, we manipulated the diets of both the focal animals and their mothers using a split-clutch design.

If the developmental history of a female (including provisioning by the mother) influences life-history and morphological traits, females reared on high quality diet should be able to allocate more resources to all life-history traits. These females may therefore invest more in choice (i.e., be more likely to express four rather than three spermathecae), and simultaneously produce larger eggs, leading to larger offspring with larger traits. Furthermore, females with good genes for resource acquisition should be better able to produce a fourth spermatheca even if the resources are limited for mothers and/or offspring. However, a trade-off in investment between choice apparatus and fecundity or other life history characters may reveal that such allocation decisions are a strong constraint on the evolution of choice.

MATERIALS AND METHODS

Experimental flies and measurements

We established selection lines with females having either three or four spermathecae (Thüler et al., unpublished, see chapter 2). Flies were randomly paired within replicate lines (but sib matings were avoided). Females of the sixth generation following the initiation of artificial selection were allowed to lay eggs on cow dung. We counted the number of laid eggs and transferred ten eggs per female singly into small plastic containers containing dung according to one of two treatments (5 eggs per treatment, two treatments per female). The control treatment featured homogenized, undiluted cow dung, while the low quality dung treatment was prepared by diluting 125g of control dung with a 150 ml of a 2% agarose solution. In pilot work this mixture effectively diluted the quality of the larval food resource. Containers

were haphazardly placed within a climate chamber and kept under controlled conditions (20ºC, 65% relative humidity, photoperiod of 12 h [L:D 12h:12h]). Freshly-emerged flies (generation 7) were kept singly in vials with *ad libitum* sugar, water and live adult *Drosophila melanogaster* until sexual maturity. The procedure was repeated to establish the last generation (generation 8). Ten eggs from singly reared females of the seventh generation were again placed singly on either high or low quality dung and kept as above. After emergence, adult flies were reared under standard conditions for 14 days, then CO_2 anaesthetised and frozen.

Males and females of the last generation were dissected and with a microscope mounted camera pictures were taken of the female and male genital tract (see Chapter 1). Spermathecal area, spermathecal duct length, accessory gland area, accessory gland duct length, testis area and sperm length were measured using the public domain image analysis software ImageJ (http://rsb.info.nih.gov/ij/). Measurements were made manually by clicking approximately 30 waypoints along the outline of the area or centre of each sperm or duct. To measure sperm head and tail length, the testes were dissected and sperm was released in a dropped of distilled water on a microscope slide. After air-drying, a drop of DAPI (4',6-Diamidino-2-phenylindole) was added to stain the DNA in the sperm head. We used the fluorescence microscope to take pictures from the stained slides. Five sperm per individual were measured using ImageJ. As an index of body size the hind tibia length (HTL) was measured (Sigurjónsdóttir 1980; Ward & Simmons 1991).

Statistical analyses

We analysed the single clutch fecundity of the focal females as a function of the selection regime, spermatheca number, HTL, dam diet, and offspring diet using a GLM (SPSS ver. 11; SPSS, Inc., Chicago, IL). We also analysed the responses of other morphological traits to selection on spermatheca number and diet manipulation (diet = dung quality). We used GLM (SPSS) with selection regimes (selection lines), replicates within selection regimes, dam diet, and offspring diet as fixed factors.

RESULTS

Female fecundity and expressed spermathecae

In general, females in 4-spermathecae-lines laid significantly larger clutches than females in 3-spermathecae-lines (table 1; Thüler et al., unpublished, chapter 2). But within lines the number of effectively expressed spermathecae (phenotype) had a significant negative effect on fecundity (effect of spermatheca expression after controlling for selection regime, table 1): females with four spermathecae in 4-spermathecae-lines laid significantly fewer eggs than flies with three spermathecae from the same genetic background. HTL showed no effect on fecundity (table 1). The manipulation of dam diet did significantly affect fecundity: dams reared on high quality dung produced larger clutches than dams reared on low quality dung (table 1). Offspring diet showed no effect.

Looking at the interactions (table 1), the effect on fecundity of selection regime interacting with dam diet was significant. This shows that within 4-spermathecae-lines dams reared on high quality dung

have larger clutches than dams reared on low quality dung. The interaction between offspring diet and spermathecae number had no significant influence on the fecundity.

In general, female reared on high quality dung express more often a fourth spermathecae than females reared on low quality dung (effect within 4-spermathecae-lines, because only there are there many females with four spermathecae and several with only three; in 3-spermathecae-lines all females expressed three spermathecae). The significant interaction between dam diet and offspring diet to effectively expressed spermathecae number showed that dam diet had a grater influence in spermatheca expression than offspring diet itself (figure 2).

Morphological traits

We also analysed the effects of selection regime, dam diet and offspring diet on several morphological traits in females and males and (see table 2 for statistics). Female offspring reared on high quality dung tend to have larger hind tibiae (HTL) than offspring reared on low quality dung; but this effect was not apparent for males. Although the selection regime had no significant effect on female and male HTL, the three-way-interaction between selection regime, dam diet and offspring diet was significant: if offspring received high quality dung, female offspring HTL was significantly larger in both 3- and 4-spermathecae-lines no matter where dams were reared (table 2, figure 3). The significance seems to be due to the offspring diet.

Selection regime had a significant effect on spermatheca size, spermathecal duct length and accessory gland duct length: while spermatheca size and spermathecal duct length were significantly larger and longer, respectively, in 3-spermathecae-lines than in 4-spermathecae-lines, accessory gland duct length was significantly longer in 4-spermathecae-lines than in 3-spermathecae-lines. The interaction between selection regime and offspring diet was also significant for spermatheca size and accessory gland duct length and showed that offspring in 3-spermathecae-lines had larger spermathecae and longer accessory gland ducts when reared on high quality dung than when reared on low quality dung (in 4-spermathecae-lines there was no effect of dung quality on these traits). Finally, dam diet had a significant effect on spermathecal duct length: they were significantly longer when dams were reared on low quality dung than on high quality dung (selection regime and offspring diet had no effect).

Concerning male traits, selection regime was the only significant predictor: sperm heads were significantly longer in 4-spermathecae-lines than in 3-spermathecae-lines; but sperm total length showed exactly the opposite: it was significantly longer in 3-spermathecae-lines than in 4-spermathecae-lines.

DISCUSSION

We studied the cost of complex female structures that may have a role in mate choice using artificial selection on spermatheca number and manipulating an environmental factor affecting condition (dam and offspring larval diet). This allowed us to separate the effects of genetic background from effects of resource acquisition, which are typically strongly confounded in any selection experiment because it is

impossible to select on one character without selecting on genetically correlated characters. Because females with four spermathecae (in 4-spermathecae-lines) may be generally better able to acquire and allocate more resources to build an extra spermathecae, we wanted to tease apart the costs of structural investment in spermathecae from any genes for high levels of acquisition that might have been selected in the 4-spermathecae lines. Using a statistical analysis that separated these factors, we showed that the number of effectively expressed spermathecae had a significant negative effect on female fecundity: if females express four spermathecae (in 4-spermathecae-lines) they have significantly smaller clutches compared to the females with three spermathecae (in 4-spermathecae-lines). This emphasizes the substantial cost of the complex reproductive tract in yellow dung flies, and underscores the need to quantify direct or indirect benefits associated with this reproductive morphology. Animals in 3-spermathecae-lines did not exhibit a decreased performance in other life-history traits. We also found a strong effect of dam diet on the expression of a fourth spermatheca, which suggests that the production of a complex choice-apparatus depends significantly on maternal condition. Although the selection treatment as a main effect was marginally non significant, its parameter estimate was very large. Further, since it is significantly interacting with dam diet we might argue that selecting up on spermatheca number leads to higher fecundity. This is presumably because of the unavoidable selection on condition that is occurring, although there is a large error associated with this parameter estimate, hence the non-significance. We discuss the implications of these findings for mate choice theory in general and for the biology of dung flies.

Mate choice, mating itself and the investment in complex choice-apparatus is costly to females and these costs of female mate choice have major impacts in evolutionary models of mate preferences (Pomiankowski, 1987; Kokko et al., 2002). Female mate preference can be influenced by condition and resource acquisition may even generate variation in mate choice behaviour (Jennions & Petrie, 1997; Hunt et al., 2005). Complex choice apparati, like the three or four spermathecae found within yellow dung flies, are costly to produce and seem also to depend on condition for their expression. The variation across females in investment in spermathecae relative to other life-history and morphological traits (such as fecundity, body size) can change with different levels of resource acquisition. These levels may explain some of the variation in spermatheca number observed in natural populations of flies.

Genetic differences between animals (e.g. in sexual traits or male ornaments) may be influenced by the quality of their environment which may be passed on to their offspring (Wolf et al. 1998), but it is not yet clear whether a expression of a certain trait is already fixed during larval development and therefore strongly influenced by maternal provisioning. Although there is considerable genetic variation in spermatheca number (Thüler et al., unpublished, chapter 2), most female dung flies may only have three spermathecae because selection for more may be weak (Ward 2000). Therefore, Ward et al. (2007) suggested that the number of spermathecae in the study population is stable because the relative benefits in offspring quality through cryptic female choice are balanced by the costs in total numbers of offspring. The balance of costs and benefits might therefore be an important factor in determining female's investment in mate choice and mate choice apparatus.

Females in 4-spermathecae-lines may have genes for high resource acquisition which has allowed them to express four spermathecae in the first place and to lay larger clutches. We therefore expect also individuals (males and females) to have larger body sizes and other reproductive and morphological traits (e.g., testes, spermathecae, accessory glands) in 4-spermathecae-lines, especially when reared on high quality dung. We only found that female body size was larger when reared on high quality dung, but no further support for expectation that individuals in 4-spermathecae-lines show larger traits. A possible explanation of the correlated response of female body size in 4-spermathecae-lines may be the ability to acquire resources because larval diet seems to be an important factor for the determination of body size. But females in 4-spermathecae-lines may face a trade-off in energy investment between life-history (clutch size) and morphological traits (body size, spermatheca size, spermathecae duct length; van Noordwijk & de Jong, 1986). Our results showed that females in 4-spemrathecae-lines have significantly smaller spermathecae and shorter spermathecal ducts than females in 3-spermathecae-lines. This may suggest a resource trade-off in size and number and length and number, respectively, and this effect was even stronger if dam diet was of low quality. But this is quite a curious result: the influence of dam diet on spermathecal duct length showed that spermathecal ducts were longer when dams were reared on low quality dung, and since selection regime had no effect, this is true for both 3- and 4-spermathecae-lines.

Condition-dependence may maintain alternative phenotypes in a population (Dawkins, 1980; Eberhard, 1982; Austad, 1984; Gross, 1984, 1996). For example, large individuals may be better fighters and thus more able to secure and defend territories (e.g. Alcock, 1979). Condition dependence is an important factor affecting investment in life-history traits (Hunt et al., 2005). But investment in one function comes at a cost of investment in other traits (Simmons & Roberts, 2005). Moreover, investment in reproductive morphology can covary negatively with investment in other traits, and this relationship has consequences for post-copulatory sexual selection and implications for sexual conflict. Ward (2000) suggests that only large females may benefit from increased spermatheca number by being able to act against male interests. Large females may have stronger muscles around the reproductive tract and females with four spermathecae may be able to manipulate the ejaculates of different males to a greater extent. Therefore large females in 4-spermathecae-lines may be better able to reduce the high fertilization success of a second mate (P2) than smaller females (low-quality female). Alternatively, the displacement of sperm within large females happens faster because of the lower spermathecal volume, which requires no special female adaptions.

Interestingly, male traits were only affected by the selection regime. Sons in 4-spermathecae-lines had shorter sperm (total sperm length) than sons in 3-spermathecae-lines; but the sperm head showed exactly the opposite. Female preferences for male traits ought to establish genetic co-variance between the female preference and the male traits that are preferred. If females with four spermathecae have a higher ability to choose sperm, spermatheca number should be genetically correlated with traits in males that affect high performance within females with four spermathecae. Testing such a hypothesis is difficult for several reasons. 1) Because the design of our selection experiment specifies random mating within lines,

linkage disequilibrium between the female preference and male traits breaks down at a rate of 50% per generation. This means the experiment is only good at finding male traits that are physically linked to genes for female preference. 2) Because of associations between female preference and other aspects of female biology (e.g., condition), it is hard to conclude that any patterns of co-variance are due to sexual selection as opposed to simple pleiotropy. In other words, it is plausible any correlated response in males might be due to condition dependence of male traits rather than due to historically non-random mating caused by female choice.

The maintenance of genetic variation in male sexually selected traits is one of the most pervasive problems in sexual selection. Fluctuating sexual selection imposed by status-dependent mate choice is important for the maintenance of additive genetic variation for sexually selected traits (Bussière et al., 2008) and provides an elegant way to account for the observable variation in mate choice in nature in spite of theoretical difficulties to account for these things especially at equilibrium (e.g., see Cameron et al., 2003). If the genes governing female mate choice are dependent on environmental conditions for their expression, as has been demonstrated in wax moths (*Achroia grisella*) reared at different temperatures (Rodriguez and Greenfield 2003), genotype by environment interactions may be an important factor maintaining genetic variation in costly female choice and thus potentially driving heterogeneity in sexual selection on male sexual traits. Nevertheless, the costs of investment in choice may be finely balanced by clutch size (number of offspring) and depend on condition (including maternal condition) so that selection on males is not strongly consistent across generations.

ACKNOWLEDGEMENTS

Many thanks to Andy and Katharine Pemberton, Ursula Briegel, Bettina Eugster and Samuel Tanner for their help in the laboratory and with the selection lines.

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FIGURES AND TABLES

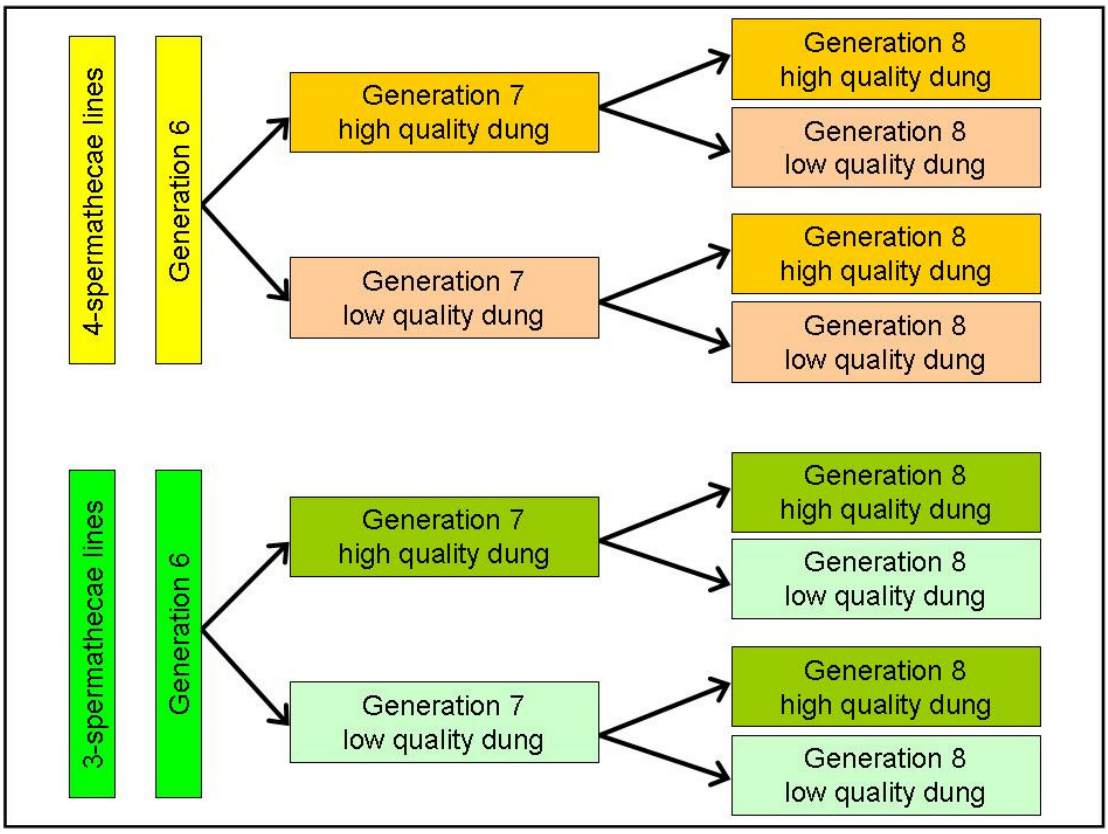

Figure 1. Design of the selection experiment. Flies of the last two generations were reared singly on different dung qualities to manipulate the dam and offspring larval diet.

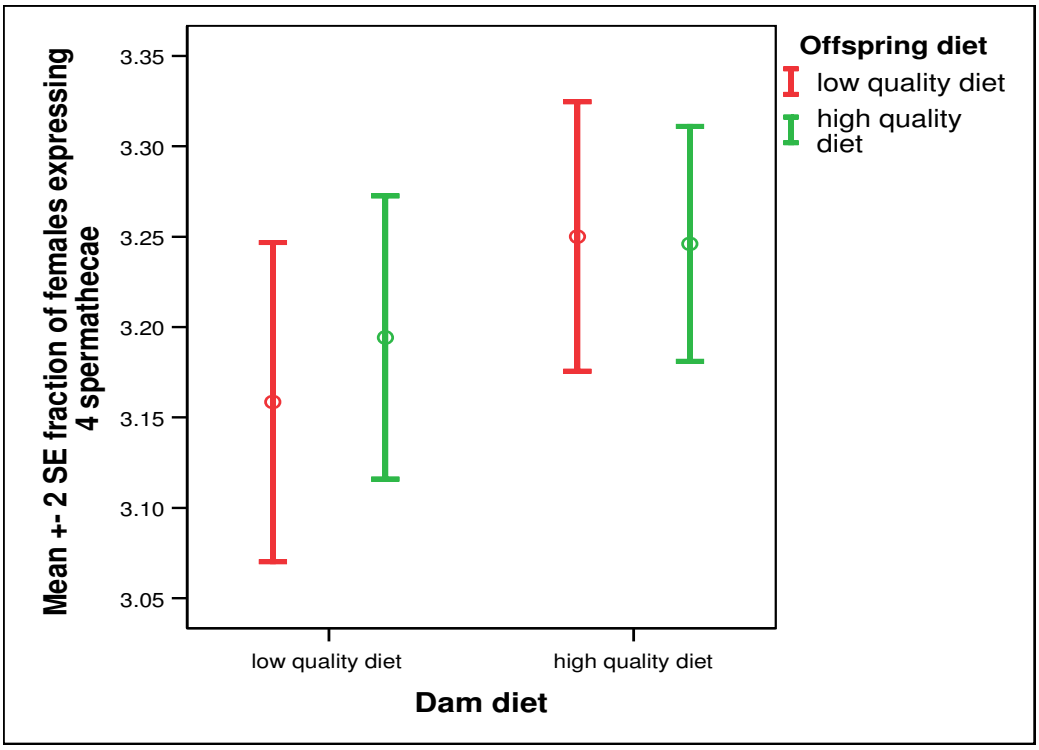

Figure 2. Differences in the expression of spermatheca number (3 or 4) in the offspring of the 4-spermathecae-lines as a function of dam and offspring diet (dung quality). Factor (y-axes): 3: females express only three spermathecae, 4: females express only four spermathecae.

Table 1: Results of GLM of female fecundity as a function of selection regime, spermatheca number, HTL, dam diet, and offspring diet.

Source	**df**	**MS**	***F***	***P-Values***
Intercept	1	2003209.862	10085.939	<0.001**
Female Hind Tibia Length (HTL)	1	73.152	.311	0.577
Selection regime	1	5774.093	29.072	**<0.001****
Dam diet	1	24403.801	122.870	**<0.001****
Offspring diet	1	7.883	.040	0.842
Spermatheca number	1	693.516	3.693	**0.055***
Female Hind tibia length (HTL) x Selection regime	1	0.532	0.071	0.790
Selection regime x Dam diet	1	1996.585	10.053	**0.002****
Offspring diet x Spermatheca number	1	415.936	2.215	0.137
Error	898	198.614		

(Significance level p<0.05*; p<0.001**; grey shaded: traits in 4-spermathecae-lines or high quality dung show higher values)

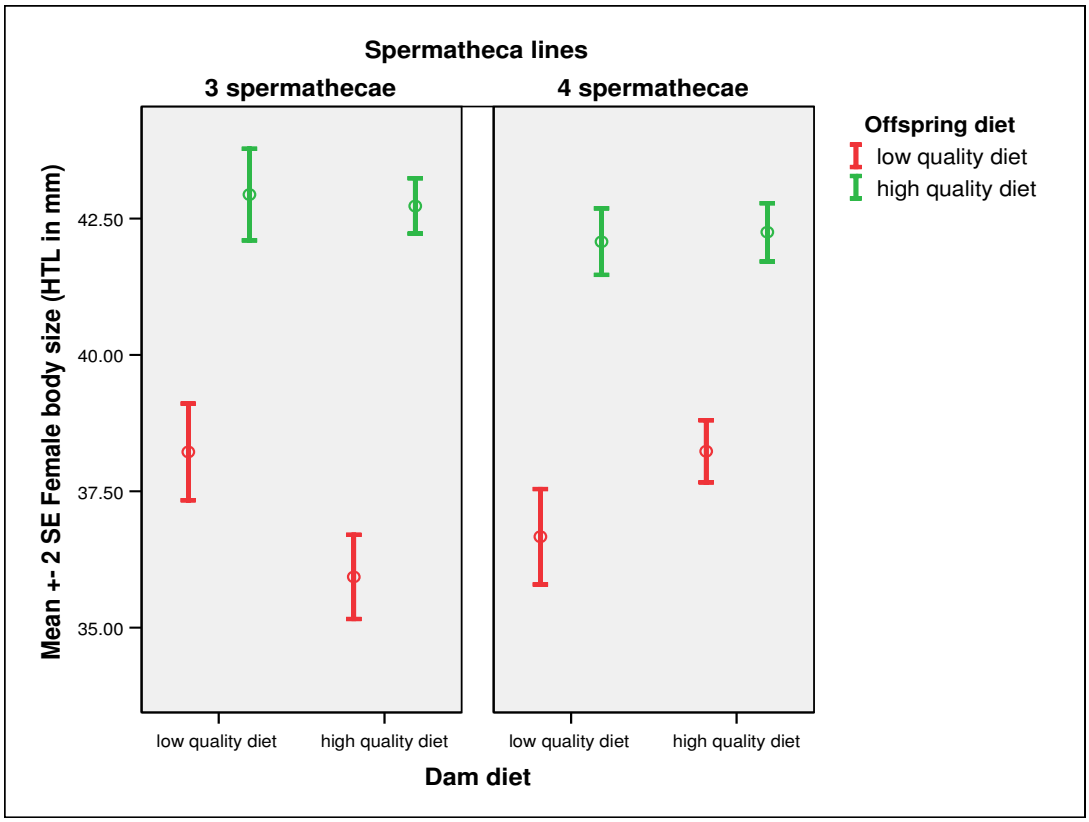

Figure 3. Female body size as a function of selection regime, dam diet and offspring diet.

Table 2. GLM of female and male body size (HTL, top) and male and female internal reproductive traits as a function of selection regime (3- and 4-spermathece-lines), dam and offspring diet (corrected for body size HTL; grey shaded: high quality dung or traits in 4-spermathecae-lines show higher values).

Source	Female Hind Tibia Length				Male Hind Tibia Length			
	df	MS	*F*	*P-Values*	df	MS	*F*	*P-Values*
Selection regime (3 or 4 Spermathecae Lines)[1]	1	0.172	0.032	0.859	1	174.113	0.106	0.746
Replicates (within Selection regimes)[1]	4	20.674	3.817	**0.005***	4	469.274	0.285	0.886
Dam diet [1]	1	0.017	0.003	0.955	1	287.285	0.175	0.678
Offspring diet[2]	1	1171.017	274.973	**<0.001****	1	204.600	3.645	0.077
Family (Dam-ID)[2]	113	6.287	1.476	**0.030***	40	26.262	0.468	0.970
Selection regime * Dam diet[1]	1	20.447	3.775	0.053	1	47.594	1.400	0.242
Dam diet * Offspring diet[2]	1	0.393	0.092	0.762	1	9.534	0.004	0.950
Selection regime * Offspring diet[2]	1	7.454	1.750	0.189	1	1422.359	0.601	0.451
Selection regime * Dam diet * Offspring diet[2]	1	25.336	5.949	**0.017***	.	.	.	.
Error 1	198	5.416			54	88818.676	.	.
Error 2	85	4.259			14	2366.938		

1: tested against error 1, 2: tested against error 2; error 2: error1-family (Dam-ID), Significance level p<0.05*; p<0.001**

Source (male traits)	Testis size				Sperm Head Length				Sperm Total Length			
	df	MS	*F*	*P-Values*	df	MS	*F*	*P-Values*	df	MS	*F*	*P-Values*
Selection regime (3 or 4 Spermathecae Lines)[1]	1	171.780	0.103	0.750	1	183.765	11.020	**0.002***	1	4823.017	10.077	**0.003***
Replicates (within Selection regimes)[1]	4	471.878	0.282	0.889	4	20.400	1.223	0.312	4	812.227	1.697	0.164
Dam diet[1]	1	260.271	0.155	0.695	1	2.345	0.141	0.709	1	232.584	0.486	0.489
Offspring diet[2]	1	1000.707	0.393	0.541	1	14.461	0.899	0.360	1	0.894	0.004	0.951
Family (Dam-ID)[2]	40	1392.198	0.547	0.928	40	16.869	1.049	0.489	40	559.445	2.433	**0.043***
Selection regime * Dam diet[1]	1	174.576	0.104	0.748	1	1.473	88.000	0.767	1	431.104	0.901	0.347
Dam diet * Offspring diet[2]	1	26.018	0.010	0.921	1	23.594	1.467	0.247	1	787.788	3.426	0.087
Selection regime * Offspring diet[2]	1	1316.489	0.517	0.485	1	26.725	1.662	0.220	1	336.009	1.461	0.248
Error 1	53	1674.814			53	16.675			53	478.619		
Error 2	13	2544.403			13	16.078			13	229.922		

1: tested against error 1, 2: tested against error 2; error 2: error1-family (Dam-ID), Significance level p<0.05*; p<0.001**

Source (female traits)	Spermatheca size				Spermathecae Duct Length				Accessory Glands Size				Accessory Glands Duct length			
	df	MS	F	P-Values	df	MS	F	P-Values	df	MS	F	P-Values	df	MS	F	P-Values
Selection regime (3 or 4 Spermathecae Lines)[1]	1	580.378	24.844	**<0.001****	1	36965.466	14.070	**<0.001****	1	55.129	0.090	0.765	1	12149.843	5.236	**0.023***
Replicates (within Selection regimes)[1]	4	71.239	3.050	**0.018***	4	3320.292	1.264	0.286	4	2518.791	4.105	**0.003***	4	4852.642	2.091	0.083
Dam diet[1]	1	4.719	0.202	0.654	1	12019.092	4.575	**0.034***	1	532.184	0.867	0.353	1	859.886	0.371	0.543
Offspring diet[2]	1	1.123	0.076	0.784	1	1056.158	0.503	0.480	1	983.808	1.733	0.192	1	2466.525	1.188	0.279
Family (Dam-ID)[2]	113	29.725	2.009	**<0.001****	113	3013.839	1.435	**0.042***	111	647.907	1.141	0.265	111	2502.244	1.205	0.187
Selection regime * Dam diet[1]	1	13.155	0.563	0.454	1	5186.651	1.974	0.162	1	350.467	0.571	0.451	1	680.404	0.293	0.589
Dam diet * Offspring diet[2]	1	5.985	0.404	0.527	1	5986.607	2.850	0.095	1	92.941	0.164	0.687	1	208.583	0.100	0.752
Selection regime * Offspring diet[2]	1	60.935	4.117	**0.046***	1	5113.780	2.434	0.123	1	27.905	0.049	0.825	1	9538.311	4.592	**0.035***
Selection regime * Dam diet * Offspring diet[2]	1	2.550	0.172	0.679	1	337.202	0.161	0.690	1	725.654	1.278	0.262	1	2.624	0.001	0.972
Error 1	197	23.361			196	2627.247			194	613.655			194	2320.319		
Error 2	84	14.799			83	2100.924			83	567.848			83	2077.021		

1: tested against error 1, 2: tested against error 2; error 2: error1-family (Dam-ID), Significance level p<0.05*; p<0.001**

FEMALE ACCESSORY GLAND FLUID PROMOTES SPERM SURVIVAL IN YELLOW DUNG FLIES

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ABSTRACT

Female and male reproductive traits coevolve through pre- and post-copulatory sexual selection and sexual conflict. Although males typically transfer large numbers of sperm during copulation, only a small proportion reach the fertilization site because females often actively or passively reduce sperm number in their reproductive tract. Males may transfer substances to protect their ejaculates against female attacks, which benefits males and can harm females. In turn, females may use accessory gland fluids to control sperm storage or paternity. Female yellow dung flies (*Scathophaga stercoraria)* have paired accessory glands, the fluids of which are involved in fertilization and egg laying. A further proposed function for these fluids is spermicide, for which there is as yet little supporting evidence. Alternatively, female accessory gland fluid may help keep sperm alive to avoid fertilization failure. We investigated the function of yellow dung fly accessory gland fluid when interacting with male sperm *in vitro*. Significantly more sperm remained alive when exposed to accessory gland fluid than when exposed to buffer only (66% vs. 47%). We conclude that female accessory gland fluid can help nourish rather than kill male sperm, but note that selective nourishment of sperm is as consistent with cryptic female choice as is selective spermicide.

Keywords: yellow dung flies, *Scathophaga stercoraria*, accessory glands, accessory gland fluid, sperm survival, sexual selection

INTRODUCTION

Most work on sexual selection has traditionally centred on explaining the often extreme sexual dimorphisms represented by elaborate male secondary sexual traits, but recent studies have extended the focus to other, less conspicuous traits such as the internal reproductive anatomy of males and females, or even the molecules involved in reproductive interactions (Civetta & Singh, 1999). It is now well established that female and male reproductive traits evolve through pre- and post-copulatory sexual selection or sexual conflict, leading to correlated evolution between the sexes in the traits involved (Dybas & Dybas, 1981; Pitnick et al., 1999; Presgraves et al., 1999; Pitnick et al., 2003). For example, in *Drosophila melanogaster* selection for longer sperm leads to changes in size of the female's spermathecae (Miller & Pitnick, 2002). Further, traits involved in pre-copulatory sexual selection may influence the evolution of traits involved in post-copulatory sexual selection due to correlated responses to selection (e.g. male eye span in sexually dimorphic stalk-eyed flies, Wilkinson et al., 2005).

In species with internal fertilization males typically transfer very large numbers of sperm, but often only a small proportion ever reach the fertilization site (Chang, 1951; Hartman, 1957; Bedford, 1970; Austin, 1975; Smith & Yanagimachi, 1990; Suarez, 1987), presumably because the reproductive tracts of females are generally hostile to sperm (Birkhead et al., 1993). Females of a variety of species actively reduce the sperm numbers in their reproductive tract by extrusion, dissolution or degradation (Davey, 1985; bruchid beetles: Eady, 1994; house flies: Degrugillier, 1985), implying female influences on sperm storage and paternity, which may or may not be adaptive. Therefore many male sperm are short lived within the female's genital tract. In response, males of several insects produce and transfer to females various accessory substances to protect their ejaculates against female enzymatic attack and digestion (Chapman, 2001; Lung & Wolfner, 1999; Lung et al., 2002; Leopold et al., 1971; Merritt, 1989; Duvoisin et al., 1999). These male substances may benefit the males to the detriment of the females, e.g. by decreasing female attractiveness or receptivity to further matings, accelerating egg laying, storage and use of sperm, or decreasing female life span (Chen, 1996; Wolfner, 1997, 2002; Chapman et al., 2001).

The female reproductive tract of insects typically includes a pair of ovaries, from which the oviducts emanate to further join and form a common oviduct, one to several spermathecae with corresponding ducts, and paired accessory glands (Wigglesworth, 1967; Gillott, 1988; Chapman, 1998; Sturm, 2008). Davey (1985) documented various functions of accessory glands: lubrication during copula and oviposition for faster mating and egg laying, production of oviposition pheromones, or protective secretions to coat eggs. In *Musca domestica,* accessory gland fluid moves along with the spermatozoa to the fertilization chambers and is used to dissolve the cap of the mature egg to allow fertilization (Leopold & Degrugillier, 1973; Leopold et al., 1978). Female accessory gland fluid may also be used to create a hostile insemination site so as to potentially facilitate cryptic female choice (Birkhead et al., 1993; Hellriegel & Ward, 1998; Hosken et al., 2001). Several studies have hypothesized that female accessory gland fluid can selectively kill sperm (i.e. act as a spermicide: Hellriegel & Ward, 1998; Greef & Parker,

2000, Hosken et al., 2001; Bernasconi et al., 2002). A female may benefit from such sperm killing by promoting male competition (Birkhead et al., 1993; Bernasconi & Keller, 2001), countering antagonistic male adaptations (Chapman et al., 1995; Rice, 1996; Andrés & Arnqvist, 2001), or simply biasing paternity in favour of males of high genetic quality (Birkhead et al., 1993; Birkhead, 1998; Hellriegel & Ward, 1998; Greeff & Parker, 2000; Bernasconi et al. 2002).

While the number of sperm stored is generally lower than the number transferred (Hosken et al., 2001; Bernasconi et al., 2002), this does not necessarily mean that sperm are actively killed by females; sperm may simply get lost in the female reproductive tract (Arthur et al., 2008). Although sperm viability varies in different parts of a female's reproductive tract in yellow dung flies, Bernasconi et al. (2002) failed to show that accessory gland fluid significantly lowers sperm viability. Therefore, there is yet no direct evidence that accessory glands debilitate sperm or contribute to sexual conflict via spermicide (Bernasconi et al., 2002). In fact, the opposite function of female accessory gland fluid is also conceivable. Killing sperm by degradation or keeping sperm alive are thus two possible but contrary functions of female accessory gland products, both consistent with female reproductive influences on paternity.

Study organism and predictions

In a study of genetic correlations between the sexes in yellow dung flies, we found a positive (albeit not significant) genetic correlation between accessory gland size and testis size (Thüler et al., unpublished, chapter 1). This positive association between the sizes of the female accessory glands and the male testes suggests female-male-co-evolution mediated by either similar (e.g. sperm storage facilitation) or opposed (e.g. sperm killing) interests. A positive association (or positive genetic correlation) may occur either if males with large testes perform better in females with large accessory glands (or *vice versa*), representing similar interests; or due to opposed interest. This may then cause an intersexual conflict (males with large testes and more sperm mate with females with large accessory glands and more fluid to kill sperm), where both traits would be selected to became even larger and may be involved in an antagonistic arm race ending up in a positive association.

There is some evidence that partitioning of sperm occurs within the female reproductive tract, potentially allowing some level of sperm choice (Otronen et al., 1997; Ward, 2000; Bussière et al., 2009 in press). Bernasconi et al. (2002) showed that sperm viability varies in different parts of a female's reproductive tract, but they were unable to demonstrate that accessory gland fluid significantly affects sperm viability. One limitation of their approach was that they used previously frozen accessory gland fluid for *in vitro* experiments.

Here we investigate the role of accessory gland fluid in the yellow dung fly using fresh accessory gland fluid to circumvent the possibility that some important substances might be inactivated by freezing. We predict contrasting observations for sperm viability depending on which, if any, of the alternative hypothetical functions for accessory gland fluids is true: we should find more (rather than fewer) live sperm after exposure to accessory gland fluid (compared to a control treatment) if accessory glands

nourish sperm. In contrast, if the glands promote spermicide, fewer sperm should be alive after exposure to accessory glands than in controls.

MATERIALS AND METHODS

Experimental flies and life/dead sperm analyses

We collected flies from a pasture near Zurich (in Fehraltorf, Switzerland) in November 2006, and reared them in the laboratory using standard conditions (Ward & Simmons, 1991). For this experiment we used offspring of the sixth laboratory generation. After flies reached sexual maturity (> 10 d after adult emergence) we dissected the females' accessory glands, extracted the fluid by rupturing it in a micro-centrifuge tube, diluted in 20µl buffer (Schneider's *Drosophila* medium; this solution is referred to as "accessory gland fluid suspension"). We did the dissection in five blocks of <12 minutes, with 10 females and 6 males per block for a total of 47 females and 94 accessory glands and 30 males to provide equally fresh tissue for all sperm. We homogenized the extracted accessory gland fluid from the 10 females per block so that all sperm samples (6 males per block) received the same dose of accessory gland product. To obtain live sperm samples, we dissected males and extracted sperm from the proximal third of one of their testes by piercing the testis and pressing it lightly with a needle until approximately one third of the testis contents was released into 100µl buffer (Schneider's *Drosophila* medium plus 10% heat-inactivated fetal calf serum; Bernasconi et al. 2002) on a glass slide (this solution is referred to as "sperm suspension").

All dissections were performed after flies had been anesthetized with CO_2. To identify whether the female's accessory gland fluid affects sperm viability, we exposed sperm from 30 individual males to either fresh accessory gland fluid mixed with buffer or to buffer alone, in a paired design such that sperm from each male was treated with and without accessory gland fluid. We incubated 15µl of the sperm suspension with 30µl of buffer plus 15µl of female accessory gland fluid suspension (or 45 µl of buffer in the control treatment for a total of 60µl) at room temperature for 11+/-2 min.

Thirty µl of the accessory gland fluid–sperm mixture, or the same amount of only sperm and buffer, were released on a micro slide and examined under a fluorescent microscope. Sperm viability was assessed using the live/dead^{TM} Sperm Viability Kit (L-7011, Molecular Probes), which consists of a membrane-permeant (live) nucleic acid stain (SYBR14, 1 mM in DMSO, diluted 1:50; emission max. 516 nm) plus a dead-cell stain (probidium iodide, 2.4 mM in water; emission max. 617 nm). After incubation, we added 5 µl of each stain, vortexed lightly and incubated the suspension in the dark for 5 min before viewing the sample under the fluorescent microscope. The fluorescent dye SYBR 14 enters only intact cell membranes (living cells), whereas the fluorescent dye promidium iodide enters only dead cells with damaged membranes. In a few cases, cells take on both stains; these doubly-stained cells were scored as dead (Bernasconi et al., 2002). The fluorescent microscope contains three filter sets, allowing the viewing and recording of digital photographs of green only, red only and green and red light to clearly distinguish dead from live sperm. The proportion of live sperm in the sample was estimated from the number of live and dead sperm (i.e. green and red sperm) at 20x magnification using 20 +/- 2 images per male. Images

were recorded 20 +/- 2 min after dissection and 9 +/- 1 min after adding the stains. For the sperm count we randomly took an image of sperm mixed with either accessory gland fluid or only buffer to estimate the proportion of live and dead sperm in each treatment. We analyzed the (arcsine square-root transformed) data using a paired t-test for 30 males with individual samples weighted by the total number of sperm per male, because not every image contained the same number of total sperm (figure 1).

Western Blot (gel electrophoresis and blotting)

We used a Western Blot to analyze the products in the accessory gland fluid, especially the presence of proteins and their size. We dissected females, extracted the accessory glands and released them into LDS- (lithium dodecyl sulfate) sample buffer (20µl, 1 accessory gland per µl). After keeping the sample with the accessory glands in an ultrasound bath for 5min to break open the cell structure and release the proteins, we centrifuged the material for 20 min at full speed to generate a supernatant with the accessory gland contents. We then mixed 10µl of the supernatant with 3µl of LDS sample buffer. As a positive control we used hen blood and prepared it the same way. After washing the gel with deionised water, we fixed the comb and subsequently filled it with MOPS- (3-(N-Morpholino)-propanesulfonic acid) buffer. With a HPLC-syringe (HPLC: high performance liquid chromatography) we then poured 10µl of the sample per slot and ran the gel electrophoresis at 200V for 50min. For the blotting we first put the PVDF- (Polyvinylidene Difluoride) membrane in methanol for 30s, then in transfer buffer for several min. After electrophoresis we arranged all components (gel, filters, membrane and sponge) and ran the blotting at 30V for 60min. With Ponceau S (sodium salt of a diazo dye to prepare a stain for rapid reversible detection of protein bands on PVDF membranes) we coloured the membrane and washed it finally with deionized water. We could now identify the protein bands and determined their size with a standard-scale.

RESULTS

Accessory gland fluid experiment - life/dead sperm

We successfully dissected 47 females out of total 50 females and 36 males to get 30 successful dissections. We totally counted 7738 sperm (buffer dead: 1819, buffer alive; 1453; accessory gland dead: 1520, accessory gland alive: 2955) and an average sperm per male of 258±53. For the sperm-counting we only used the green and red filter, because this was sufficient to clearly distinguish dead from live sperm.

The percentage of live sperm was much greater when exposed to accessory gland fluid compared to only buffer ($t_{29} = 5.843$, $P < 0.001$; Buffer + live sperm: Mean ± SD 46.55% + 14.41 sperm alive, SE: 2.63; accessory gland fluid + live sperm: Mean ± SD 65.64% + SD 13.70 sperm alive, SE: 2.50; figure 1).

Western Blot

Although the blotting was successful (because we could detect the standard-scale), we were not able to detect any protein bands and therefore any substances contained in accessory gland fluid. The reason could be that the concentration of proteins in the accessory gland fluid was too low.

DISCUSSION

Many studies have confirmed that accessory gland fluid is involved in several reproductive processes (e.g., fertilization, egg laying, egg coating, lubrication; Leopold & Degrugillier, 1973; Leopold et al., 1978; Greef & Parker, 2000; Hosken et al., 2001; Bernasconi et al., 2002). For example, accessory glands in female *Musca domestica* produce substances that are involved in dissolving the cap substance from mature ovarian eggs (Degrugillier, 1985), but this secretion was mainly involved in fertilization and sperm degradation (Leopold & Degrugillier, 1973; Leopold et al., 1978; Birkhead et al., 1993; Hellriegel & Ward, 1998; Hosken et al., 2001).

The sexes have different reproductive interests, potentially resulting in sexual conflict (Chapman et al., 2003; Pischedda & Chippindale, 2006). By providing a hostile environment, females could easily kill some incoming sperm and transfer (actively or passively) the remaining sperm to the spermathecae (Hellriegel & Ward, 1998). Such sperm killing could be a mechanism to reduce problems associated with genetic incompatibility (such as in hermaphrodites and gonochorists: Bishop, 1996; Olsson et al., 1996b; Stockley, 1999), but selective spermicide could also provide females with another mechanism to influence paternity, which would be an example of post-copulatory cryptic female choice (Birkhead, 1998). Further, it is possible that accessory gland fluid components (in the function of spermicide) are female adaptations that arose in the context of sexual conflict, in the same way that male substances transferred during copulation can influence a female's behaviour and decrease her lifetime reproductive success (e.g. Chapman et al., 2003).

Although Bernasconi et al. (2002) showed that after mating sperm viability was significantly lowered in spermathecae compared to a male's testes, their *in vitro* exposure of sperm to several parts of the female reproductive tract, including accessory glands, showed no sperm degradation. Rather than producing substances hostile to sperm, we here found that the accessory glands of female yellow dung flies produce substances that apparently increase sperm survival, as the proportion of live sperm counted in accessory gland fluid was much higher than in buffer. Sperm are often short lived within a female's tract, but it is not clear if they are short lived *per se* or get killed by females, since their viability varies in different parts of the female reproductive tract (Bernasconi et al., 2002). Any sperm mortality may either be a result of individual differences in sperm viability (variation in individual sperm quality, motility and longevity; Bernasconi et al., 2002) or due to female influences (e.g. accessory gland fluid). Our experiment supports the latter mechanism.

The amount of sperm males transfer to the females during copulation varies with copula duration (Parker & Simmons 1994), and the effect of accessory gland fluid might change with the ratio of sperm-to-fluid. As we diluted the accessory gland fluid in buffer according to Bernasconi et al. (2002), we may also have diluted any effect accessory gland fluid may have on sperm. However, it is highly unlikely that the net effect of accessory gland fluid on sperm reverses depending on the concentration of the fluid.

We hypothesized that accessory gland fluid may contain different components such as proteins with different functions as found in male *Drosophila* (accessory gland proteins, e.g. Chapman et al., 2000;

Moura et al., 2006). Although our Western Blot was successful (because we could at least detect the standard-scale), we were not able to detect any protein bands and therefore any substances contained in accessory gland fluid. Leopold & Degrugillier (1973) showed in house flies that removal of the accessory glands inhibited penetration of the eggs by sperm. This indicates that the accessory gland fluid might activate sperm or alter the permeability of the egg membrane before fertilization takes place. Hosken et al. (2002) further showed for *S. stercoraria* that gland extract does not inhibit bacterial growth, suggesting that accessory gland fluid is more likely involved in fertilization functions rather than antimicrobial immunological processes.

We note however that selective provisioning of sperm could in principle serve the same function as selective spermicide, by creating conditions of sperm storage that favour some males over others. But whether a sperm nourishing function of accessory gland fluid can act to actively favour certain ejaculates over others remains unclear. As a general principle across taxa, the costs and benefits of differential spermicide versus sperm provisioning are likely to depend on the specific circumstances of the mating system in question. There is evidence that yellow dung fly females actively store sperm from different males separately in their spermathecae (Otronen et al., 1997; Bussière et al, in press), but the actual processes involved are not yet clear and we found no evidence in yellow dung flies for a directly spermicidal function of accessory gland products. However, because keeping sperm alive for weeks inside the reproductive tract may be energetically costly for females, females may benefit from selective nutrient provisioning of sperm. Females should store only as many sperm as are needed in the short term, and kill or absorb any unnecessary or unfavoured sperm (Birkhead, 2000). In yellow dung flies copulating again to obtain sperm is no problem for females, because there are always many males around the oviposition site (Parker, 1979).

The possible role of sperm viability in sexual selection in yellow dung flies will require further study. Our study clarifies the influence of accessory gland fluid on sperm viability, but we cannot exclude the possibility that other organs or their secretions may also affect sperm viability either inside the spermathecae or in other parts of the female reproductive tract. More work on the physiological and biochemical interactions involved in sperm storage and use as well as the reproductive consequences of sperm mortality for male fertilization success and female fitness is needed before this possibility can be thoroughly evaluated.

ACKNOWLEDGEMENTS

We thank Ursula Briegel for help in the lab, and Andy Pemberton, Barbara Hellriegel and Giorgina Bernasconi for useful comments on the protocol and methods.

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FIGURE

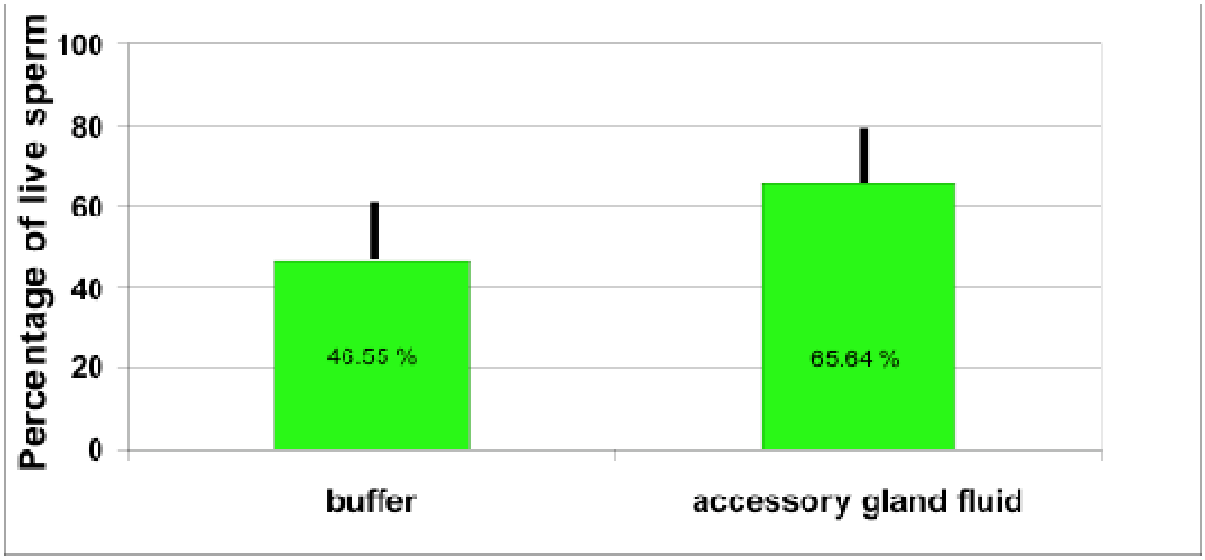

Figure 1. Percentage of live exposed to buffer or accessory gland fluid. Buffer: live sperm: 46.55%, SD: 14.41, SE: 2.63; Accessory gland fluid: live sperm: 65.64%, SD: 13.70, SE; 2.50.

ACKNOWLEDGEMENTS

I like to thank very much…

Paul Ward for being my supervisor and giving me the opportunity to accomplish a dissertation in his lab and for interesting discussions and suggestions on my experiments;

Luc Bussière and Wolf Blanckenhorn for supervising my thesis, statistical advice, English corrections, constructive comments and extensive discussions that greatly improved the manuscript and especially for moral support and encouragement;

Erik Postma for work on my data with ASReml and helpful comments on my thesis;

Lukas Keller for support and discussions of my work;

Ursula Briegel, Yves Choffat, Rosemarie Keller and Sabine Marti for providing me with lab material, secretary services and computer support;

Andy and Katharine Pemberton, Samuel Tanner, Bettina Eugster and Ursula Briegel for helping with the selection lines, feeding and dissecting flies;

Julia Burgener, Fabienne Di Gennaro, Sandra Gartner, Rahel Leugger, Barbara Schnüriger and Manuela Varini for feeding flies;

Roland Gautier, Gioia Schwarzenbach, and Thomas Honegger for giving helpful advice in the lab;

Katharina Foerster for helpful advice on statistics (ASReml);

Giorgina Bernasconi and Barbara Hellriegel for helpful comments on the accessory gland experiment;

Further, I like to thank very much…

Andy and Katharine Pemberton, Luc Bussière, Sonja Sbilordo, Debbie Zulliger and Peter Wandeler for their moral support and encouragement and endless discussions;

My parents Romy and Hanspeter Thüler, my sister Daniela Thüler and my close friends Sonja Kunz, Janine Stüssi and Roger Brandenberger for their patience, moral support and encouragement;

All my colleagues from the Zoological Museum for being part of the group.

Without all their great help, this work would not have been possible!

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